# UNDERSTANDING THE REGULATION OF ADIPOGENESIS AND ADIPOCYTE METABOLISM IN OBESITY

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## ABSTRACT

Inflammation in hypertrophic adipose tissue is a critical inducer of adipose tissue dysfunction, which ultimately leads to systemic insulin resistance and type 2 diabetes. Elevation of lipopolysaccharide (LPS) induced by high fat diet has been recently proposed to be one of the potential mechanisms contributing to the development of inflammation and metabolic dysfunction of adipose tissue in obesity. Therefore, it is of interest to expand our understanding of LPS effect on adipocyte metabolism and to identify the molecular mechanism by which LPS deteriorates adipose tissue metabolism. In the first study, we investigated the effect of LPS on the adipogenic capacity and cellular senescence of adipocyte progenitors by using stromal-vascular (SV) cells isolated from inguinal adipose tissue of C57BL/6 mice. We found that LPS treatment for 24-hour prior to the induction of differentiation led to the inhibition of adipogenesis. In addition, LPS induced features of premature senescence of SV cells. Further studies showed that LPS treatment caused a reduction in Zfp423 and PPARγ expression in SV cells, suggesting that LPS impairs pre-adipocyte differentiation. In the second study, we explored the role of endosomal/lysosomal protein NPC2 (Niemann-Pick disease, Type C2) in regulating lysosomal activity and in mediating LPS effect on adipocyte inflammation and function. NPC2 knockdown reduced lysosomal protease cathepsin B levels and impaired autophagy-lysosomal activity in 3T3-L1 adipocytes. Interestingly, NPC2 knockdown diminished LPS effect on inflammatory response and blunted LPS-induced glucose uptake in adipocytes. In the third study, we determined the effect of eicosapentaenoic acid (EPA) on promoting metabolic health of adipocytes, specifically the browning of subcutaneous white adipocytes. When added to SV cell cultures during

8-day adipocyte differentiation, EPA significantly increased the expression of thermogenic genes as well as mitochondrial DNA content. These results indicate that EPA enhances energy expenditure capacity by recruiting beige adipocytes. In summary, we have demonstrated a new mechanism by which LPS disrupts adipogenesis and adipocyte metabolism. We have also characterized the role of NPC2 as an important molecular mediator of LPS-induced adipocyte inflammation and the effect of EPA on promoting the browning of subcutaneous adipocytes.

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# CHAPTER 1

# LITERATURE REVIEW

Ming Zhao wrote this chapter.

The prevalence of obesity has been increasing dramatically and globally in the last decades [1]. It's very likely that the global rates of adulthood obesity will be continuously increased in the future. The rising incidence clearly maintains the obesity as a top health concern since adulthood obesity, especially abdominal obesity, is well-established as a risk factor for a series of diseases, including cardiovascular disease (CVD), type 2 diabetes (T2D), nonalcoholic fatty liver disease (NAFLD), and some types of cancer [2].

Type 2 diabetes (T2DM) is now affecting over 20 million Americans and predicted to be the leading killer by 2050 [3]. People with uncontrolled insulin resistance will progress to T2DM, which largely increases the risk of diabetic complications such as cardiovascular disease, lower limb amputations, non-traumatic blindness, and kidney failure. Among insulin responsive tissues/organs, such as liver, skeletal muscle and adipose tissue, adipose tissue is believed to play a central role in the regulation of systemic insulin sensitivity [4]. Adipose tissue is the major site of lipid metabolism and adipokines/cytokine production. Adipose tissue dysfunction, which occurs in obesity, is the major contributor to obesity-associated metabolic complications such as hypertension, cardiovascular disease, inflammation, insulin resistance and type 2 diabetes.

# 1. Role of adipose tissue in metabolism

## 1.1 White adipose tissue (WAT)

It has long been known that white adipose tissue (WAT) plays a crucial role in regulating energy metabolism and insulin sensitivity. WAT is the main storage site of excess energy derived from food [5]. Dietary energy from glucose and lipids are stored mostly in a single large lipid droplet in the form of triglyceride in white adipocytes. During starvation or exercise, stored triglycerides are rapidly hydrolyzed and fatty acids are released as an energy source and transported to other tissues for oxidation. More importantly, adipose tissue has been recognized as an endocrine organ, and it secretes multiple biologically active peptides and hormones such as leptin, adiponectin, resistin, retinol-binding protein 4 (RBP4), and lipocalin 2 [6-9]. These adipokines have been demonstrated to be important regulators of insulin sensitivity and energy metabolism.

## 1.2 Brown adipose tissue (BAT)

In contrast to WAT, brown adipose tissue (BAT) is the major depot for thermogenesis and energy expenditure [10]. Previously, BAT in human was believed to present at interscapular region only in infants and gradually disappear with age. In recent years, active BAT has been identified in the cervical, supraclavicular, axillary, and paravertebral regions in adult humans by $^{18}$F-FDG-positron emission tomography/computed tomography (PET/CT) scanning [11, 12]. This finding strongly

reinforces our interest in the role of BAT in thermogenesis and energy expenditure in human obesity.

It's been well known that uncoupling protein-1 (UCP1) is the key marker of BAT that plays a key role in dissipating chemical energy in the form of heat for non-shivering thermogenesis. UCP1 protein expression in BAT is increased in response to cold stimulation. Sympathetic nervous system (SNS) is the major regulator of UCP1 expression and BAT activity through activating β-adrenergic receptor (β-AR) signaling pathway [13]. β-AR signaling pathway regulates the enzymatic machinery for lipolysis and fuel utilization. The β-adrenergic signaling pathway activates two downstream targets, cAMP-dependent protein kinase (PKA) and mitogen-activated protein kinase (MAPK) pathways; both pathways regulate UCP1 expression and lipolysis [14]. In brown adipocytes, the activation of these kinase pathways controls the transcription of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) which regulates mitochondrial biogenesis and UCP1 expression. In addition, there are many other molecules and transcription factors involved in the regulation of brown adipocyte differentiation and thermogenesis, including PRD1-BF1-RIZ1 homologous domain-containing 16 (PRDM16), CCAAT/enhancer-binding protein beta (C/EBP-β) and bone morphogenetic protein 7 (BMP7) [13].

The heat production in brown adipose tissue results from both lipid and glucose/pyruvate combustions. Therefore, the activation of BAT theoretically causes a negative energy balance, which could be an attractive therapeutic approach for obesity and metabolic syndrome. In previous studies the activation of BAT has been shown to

prevent weight gain in mice. In addition, BAT activity was also found to be negatively correlated with BMI and body fat percentage in humans [15]. Beyond the classical thermogenesis in response to cold, it has been suggested that UCP1-mediated heat production is also involved in diet-induced thermogenesis, playing a role in preventing the development of obesity. In the 1970s, Rothwell and Stock observed that rats fed a cafeteria diet (high in fats and sugars) gained less body weight than expected based on caloric intake, and they proposed that excess calories were being burned off by the induction of BAT thermogenesis [16]. Indeed, a number of studies proved there was an increased Ucp1 mRNA and protein expression in brown adipose tissue of mice and rats after a high-fat diet (HFD) feeding [17]. Other studies using UCP1-ablated mouse models demonstrated that diet-induced thermogenesis is dependent on brown adipose tissue activity. In the absence of thermogenic activation of brown adipose tissue, mice develop obesity spontaneously [18].

Intriguingly, findings from recent investigations demonstrated another type of thermogenic adipocyte, brown-like adipocytes (beige adipocytes) that can be recruited in WAT, particularly in subcutaneous (SC) WAT [19, 20]. This process is often referred to as browning of white adipose tissue. Beige adipocytes share many biochemical characteristics with classical brown adipocytes, including enriched mitochondria, multilocular lipid droplets and expression of UCP1. However, they originate from distinct cellular lineages and express some unique cell markers, such as Cd137, Tbx1, Tmed26 and Cited1 [20-22].

Studies have been performed to compare the gene expression profile in BAT between human and rodents. The results showed that the interscapular BAT of human infants is composed of adipocytes expressing a gene signature of classical brown adipocytes [21], while UCP1-positive adipocytes from adult human BAT possess molecular signatures that are similar to murine beige adipocytes, rather than classical brown adipocytes [20, 21], suggesting that BAT in adult humans may not be classical brown adipocytes, but results from the browning of WAT. In animal studies, it has been demonstrated that chronic cold exposure, exercise, and endocrine hormones (BMP4&7, FGF21and VEGF) can induce the development of beige adipocytes, and this browning process is sufficient to alter energy expenditure and glucose homeostasis [23]. Similar results have been observed in adult humans; cold-induced BAT activation in human adults increases energy expenditure by 10% to 27%, or 150 to 300 kcal a day [24-26]. As a consequence of cold exposure, the whole-body glucose disposal, plasma glucose oxidation, and insulin sensitivity were improved [24].

### 1.3 Regulation of adipogenesis

In adipose tissue, mature adipocytes die constantly and are replaced by new adipocytes in order to maintain adequate lipid storage capacity. In humans, roughly 10% of adipocytes are turned over every year, whereas in mice, 0.6% of adipocytes are renewed each day [27, 28]. Adipogenesis refers as to the process where adipocyte progenitors are differentiated into mature adipocytes. Adipogenesis can be briefly divided into two phases: determination and terminal differentiation [29]. During the

determination phase, mesenchymal stem cells are committed to the adipose lineage and become preadipocytes in response to appropriate adipogenic stimulation. Terminal differentiation, on the other hand, describes the process by which preadipocytes acquire the characteristics of the mature adipocytes [29]. 3T3-L1 cell line has been commonly employed as a cellular model to study adipogenesis. Since 3T3-L1 cell line has already been committed to the adipocyte lineage, most of the knowledge acquired from studies using 3T3-L1 cells is on the regulation of the terminal differentiation process. However, little is known about the regulation of determination of adipogenesis, particularly *in vivo*.

Loss- and gain-of function studies on the terminal differentiation of adipocytes revealed that PPARγ is the "master regulator" of fat cell formation, as it is both necessary and sufficient for adipogenesis. First, it has been known that mutations of PPARγ in human adipocytes showed severe lipodystrophy [30]. Second, in the gain of function studies, overexpressing PPARγ in non-adipogenic fibroblasts and myoblasts is able to induce these cells to differentiate into mature adipocytes [31, 32]. The C/EBP family members are also important transcription factors controlling adipogenesis. C/EBPβ and δ act during the early stage of adipocyte differentiation on the induction of the expression of PPARγ and C/EBPα. Finally, a positive feedback loop between PPARγ and C/EBPα promotes the expression of adipogenic genes and stimulates lipid accumulation [33, 34].

There has been recent progress in identifying transcription factors involved in adipose determination. BMP4 is secreted by differentiated adipose cells [35] and can, in a paracrine fashion, target uncommitted precursor cells in adipose tissue, leading to their adipogenic commitment. Additionally, Zfp423 has been identified as a transcriptional

determinant for the embryonic fibroblast commitment to the adipose lineage [36]. Zfp423 has been shown to induce adipose lineage commitment by amplifying the effects of BMPs via binding a SMAD-interaction domain.

Soon after the discovery of the browning process in white adipose tissue, certain signaling pathways that regulate the browning of WAT have been unveiled. Some novel cytokines, including irisin, FGF21, BMPs and meterorin-like protein as well as the classic β-adrenergic stimulation have been determined as important inducers of beige adipocyte development [37-40]. These factors upregulate the expression of multiple transcription factors, co-activators, and other molecular regulators, including PRDM16, PGC1α, CIDEA and c/EBPβ [41-43], and positively regulate the browning of WAT.

## 2. Adipose tissue dysfunction in obesity and insulin resistance

Insulin is a major regulator of energy metabolism, with a wide range of actions, including enhancing glucose uptake and triglyceride synthesis, inhibiting lipolysis in adipocytes, and suppressing hepatic gluconeogenesis and glycogenolysis, all of which tend to reduce blood glucose and lipid levels.

The molecular pathway that mediates insulin stimulated glucose uptake has been well studied. When insulin binds to the insulin receptor on the cell membrane, the insulin receptor is autophosphorylated at its tyrosine residues leading to the activation of its tyrosine kinase activity [44]. The insulin receptor then phosphorylates tyrosine residues on the insulin receptor substrates (IRSs), which then serve as docking sites for SH2-

containing enzymes such as PI-3 kinase. This leads to linear signaling cascades that result in Akt activation. As the consequence of Akt activation, GLUT4 translocation and glucose uptake into skeletal muscle and adipose tissue are enhanced; glycogen synthesis in the liver is stimulated [44]. Thus, insulin signaling plays an important role in metabolism in multiple insulin responsive tissues.

Insulin resistance (IR) is the major cause of type 2 diabetes. The mechanisms of insulin resistance have been studied intensively for decides. Currently, it's well accepted that insulin resistance is closely related with energy imbalance and obesity. Many molecular mechanisms are proposed, including ER stress, oxidative stress, dysregulation of lipid homeostasis (including FFA homeostasis), mitochondrial dysfunction, hypoxia and others [45]. Among these proposed mechanisms,, the inflammation theory is most extensively studied and widely accepted. It well explains the initial local pathology in adipose tissue and the following systemic metabolic dysregulation in insulin resistance. Some details are described in the following sections.

### 2.1 Inflammation links obesity and insulin resistance

The role of inflammation in the pathogenesis of obesity-associated metabolic disorders was first recognized by a remarkable finding in 1870s showing that the nonsteroidal anti-inflammatory drug sodium salicylate improved glycosuria in T2DM patients [46]. After decades of research, several general processes in obesity-induced adipose tissue inflammation have been established including: a) the adipocyte expansion

and dysfunction, b) the infiltration and activation of immune cells, and c) the development of insulin resistance. Details are provided below:

**a. Adipocyte expansion and dysfunction:**

White adipocyte is the major cell type that stores excess energy derived from food [5]. Dietary energy from glucose and lipids are stored mostly in a single large lipid droplet in the form of triglyceride in white adipocytes. Therefore, excessive intake of macronutrients leads to increased storage of energy in lipid droplets of adipocytes, thereby increasing lipid droplet/adipocyte size and promoting adipose tissue expansion. The enlarged adipocytes lose their normal function and have an altered secretory pattern with a decrease in the secretion of anti-inflammatory adipokines such as adiponectin and an increase in the secretion of pro-inflammatory adipokines/cytokines and chemokines such as leptin, resistin, tumor necrosis factor alpha (TNFα), interleukin (IL)-6, and MCP-1. These secretory proteins induce the infiltration and accumulation of immune cells and inflammation in the adipose tissue [47, 48].

In addition, adipocyte hypertrophy creates a local hypoxic state, which induces the secretion of HIF1, pro-inflammatory cytokines and chemokines and causes M1 macrophage infiltration and adipose tissue remodeling. Hypoxia also leads to overproduction of extracellular matrix components in obese adipose tissue [49], which in turn cause adipose tissue fibrosis.

**b. Immune cell infiltration and activation:**

Almost all different types of immune cells have been found to reside in adipose tissue, including macrophage, neutrophils, eosinophils, dendritic cells, and T and B lymphocytes. In obesity, more immune cells are recruited into white adipose tissue and they are switched to pro-inflammatory state, leading to the development of chronic low-grade inflammation in adipose tissue. Among infiltrated immune cells, macrophages have been well accepted to play a key role in chronic adipose tissue inflammation.

The dominant macrophages residing in healthy adipose tissue are M2 macrophages, which have an anti-inflammatory role. During obesity, chemokines and cytokines from adipocytes recruit monocytes into adipose tissue [50]. In addition, hypertrophic adipocytes die at some point. This attracts a vast number of macrophages into adipose tissue to clear the dead cells, forming the "crown-like structure" in hypertrophic adipose tissue [51]. The infiltrated macrophages are differentiated to M1 macrophages, which are considered as pro-inflammatory macrophages and become the major source of substantial amount of pro-inflammatory cytokines, such as TNFα and IL-6 [50]. Previous studies have shown that the majority of pro-inflammatory cytokines in obesity are produced by macrophages [52, 53]. These cytokines recruit other immune cells into adipose tissue and induce the phenotypic switch of these cells.

Besides macrophages, recent studies have demonstrated that other types of immune cells also contribute to the development of inflammation and insulin resistance in adipose tissue. Neutrophils are considered as one of the first immune cells that migrate into adipose tissue during obesity, since their numbers are increased within the first week

of high fat diet feeding [54]. Neutrophils secrete cytokines to recruit the second wave of immune cells, such as macrophages.

Eosinophils produce many anti-inflammatory cytokines (such as IL-4, IL-10, IL-13 and TGF-β) and their numbers in adipose tissue are decreased in obesity. Under the normal chow-diet condition, ~90% of IL-4-producing immune cells in adipose tissue are eosinophils [55]. Disruption of IL-4 signaling pathway by knocking out Stat6 increases insulin resistance in mice [56, 57].

Immune cells that are involved in adaptive immune responses such as T cells and B cells are also found to be altered in number in adipose tissue during obesity and play an important role in insulin resistance. Multiple studies reveal that pro-inflammatory cells including Th1 CD4 T cells, CD8 T cells and B cells were increased, while anti-inflammatory cells such as regulatory CD4 T cells (Tregs) and Th2 CD4 T cells were decreased in adipose tissue in obesity [58-60]. These obesity-inflamed immune cells play a role in initiating the secondary wave of cytokine production by recruiting and activating other types of immune cells such as macrophages.

**c. The development of insulin resistance:**

Infiltrated immune cells in expanded white adipose tissue further deteriorate the secretory function of adipose tissue, leading to the increased secretion of cytokines, such as TNFα, IL-1, RBP4 and interferon-γ [61, 62]. These cytokines could impair the insulin signaling pathway in adipocytes. TNFα is the first identified cytokine that could induce insulin resistance. In 1993, Hotamisligil et al. demonstrated that TNFα level was

increased in obese adipose tissue (AT) and that TNFα could directly induce insulin resistance in adipocytes [63]. Now, many other pro-inflammatory cytokines have been demonstrated to have similar effects. We will use TNFα as an example to explain the detailed mechanisms of cytokine-induced insulin resistance in section 2.2.

In addition, these pro-inflammatory cytokines were found to increase adipocyte lipolysis and induce adipose tissue fibrosis, which reduce the lipid-storing capacity in adipose tissue [49, 64]. Thus, excessive lipids converted from surplus energy are forced to deposit in non-adipose tissue such as liver, muscle and pancreas, which leads to systemic lipotoxicity and insulin resistance.

### 2.2 TNFα as a major regulator of adipocyte metabolism and insulin resistance

As mentioned above, TNFα is one of the most important key modulators of adipocyte metabolism, with a direct role in several insulin-mediated processes, including glucose homeostasis and lipid metabolism [65]. The TNFα levels are increased in obese adipose tissue [66]. TNFα is well known to induce insulin resistance by attenuating insulin signaling and inhibiting adipogenesis, thereby compromising metabolic functions of adipocytes. For instance, TNFα attenuates insulin-stimulated glucose and lipid uptake, decreases oxidation of glucose and lipid, increases lipolysis and de novo fatty acid synthesis, and inhibits free fatty acids (FFA) esterification to triglyceride. All these changes lead to increased fatty acid output from adipocytes, causing the elevation of circulating FFAs and systemic insulin resistance. Blocking TNFα using TNFα

neutralizing antibody or TNFα deficiency protects mice from high-fat diet-induced insulin resistance [63, 67], suggesting that TNFα plays a key role in linking inflammation to insulin resistance.

**a. TNFα pathway**

TNFα mediates its biological effects in adipose tissue via two distinct cell surface receptors: tumor necrosis factor alpha receptor 1 (TNFR1) and tumor necrosis factor-alpha receptor 2 (TNFR2) [68]. TNFR1-induced signals are required and sufficient for TNFα-induced insulin resistance [69, 70]. The binding of TNFα to TNFR1 leads to the recruitment of several adapter complexes to the trimetric death domain of TNFR1 (TNFR1-DD). As a consequence, two major downstream pathways are activated. One is the nuclear factor-kappa B (NF-κB) pathway (by activating IKK-β, which degrades I-κB) [71]. The other pathway is the mitogen-activated protein kinase (MAPK) cascades, including extracellular signal-regulated protein kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK) [72, 73]. In addition, several other signaling pathways are activated by TNFR1 and TNFR1-DD, such as protein kinase A (PKA), CREB, protein kinase C (PKC), PI3K and calcium release [68].

TNFα affects adipocyte metabolic function at multiple levels. One of the mechanisms that mediate TNFα effect is through inhibiting PPARγ. PPARγ is a master regulator of adipogenesis and lipid metabolism. By activating NF-κB, JNK and ERK activity, TNFα inhibits PPARγ transcription and activity [74-76]. The inhibition of PPARγ in turn suppresses C/EBPα, insulin receptor (IR), insulin receptor substrate-1 (IRS-1) and GLUT4 expression [77, 78], which are required for insulin-stimulated

glucose uptake in adipocytes. In addition, by activating inhibitor κB kinase-β (IKK-β), TNFα induces serine phosphorylation of IRS-1, which inhibits insulin-stimulated tyrosine phosphorylation of the IR and IRS-1 [79, 80].

**b. Regulation of lipid metabolism in adipocytes by TNFα**

TNFα inhibits insulin action on glucose uptake and oxidation, FFA uptake and lipogenesis, and inhibition of FFA release via lipolysis by regulating the above mentioned signaling pathways. Moreover, TNFα impairs the lipid storage capacity of adipose tissue by suppressing the recruitment and differentiation of new adipocytes from precursor cells by inhibiting the induction of PPARγ and C/EBPα expression [81]. In this way, TNFα action leads to the development of dyslipidemia, which contributes to systemic metabolic complications.

TNFα also regulates the production and secretion of cytokines and adipokines from adipose tissue. By regulating the production of other pro-inflammatory cytokines (e.g. IL-6 and IL-1), TNFα further amplifies its effects on peripheral tissues. Adiponectin enhances insulin sensitivity and improves the serum lipid profile through activating AMPK and increasing fatty acid oxidation in muscle [82]. The production of adiponectin is suppressed by TNFα as a result of the suppression of PPARγ and C/EBPβ and/or JNK activation [83-85], contributing to the mechanism for TNFα-induced dyslipidemia and systemic insulin resistance.

Consistent with the inhibition of fatty acid oxidation, TNFα treatment in adipocytes also leads to morphological changes in the mitochondrion, together with

downregulating the expression of genes that encode components of complex I-V in the electron transport chain [86]. The mitochondrial dysfunction by TNFα is reflected by decreased mitochondrial membrane potential and reduced production of intracellular ATP as well as accumulation of significant amounts of reactive oxygen species (ROS) [87]. Additionally, oxidative stress may in turn promote increased production of TNFα under adverse metabolic conditions [88].

**c. TNFα in BAT thermogenesis**

The previously mentioned metabolic changes in WAT led by TNFα are the potential mechanisms contributing to insulin resistance. Contradictorily, TNFα seems to increase BAT thermogenesis, which is similar to the effect of HFD in BAT. This TNFα-induced thermogenesis in BAT could be the mechanisms by which body prevents excessive weight gain and obesity. Indeed, a similar effect that TNFα increases energy expenditure has been reported in cancer cachexia [89]. More specifically, mice receiving a single intravenous injection of TNFα resulted in significant, but transient (24-48 hours) reductions in food intake and body weight, and increases in rectal temperature, resting oxygen consumption (VO2) and brown adipose tissue (BAT) thermogenic activity [90]. Similar effect of TNFα was reported in another study, including elevated rectal temperature by 0.7 degree as well as increased serum leptin in response to TNFα injection [91]. In addition, BAT UCP1 mRNA expression was increased by 1.2-fold 6-hour after the TNFα treatment, but decreased by 0.8-fold 16-hour after the treatment. The levels of UCP2 mRNA expression were also increased in BAT, WAT, and muscle 16-hour after TNFα treatment [91]. In a third study, a significant increase in UCP2 (242%)

and UCP3 (113%) gene expression was observed in skeletal muscle of mice with TNFα injection [92].

However, contradictory results were reported in other studies focusing on the chronic effects. Brown adipocytes treated with TNFα for 48hour showed a slight reduction in UCP1 expression [93]. TNFR1 KO mice fed a HFD had higher UCP1 expression in BAT, indicating the TNFα reduces UCP1 expression [94]. In addition, a chronic TNFα elevation has more complicated impact, and it may compromise BAT thermogenesis by promoting BAT atrophy or by impairing BAT differentiation [95]. Therefore, it's likely that the effect of TNFα on BAT thermogenesis may depend on its dose and duration.

In the mechanistic studies, TNFα has been shown to activate several different signaling pathways such as extracellular-regulated kinases (ERKs), JNKs, p38 MAPKs or NF-κB [93]. Some of the pathways such as p38MAPK upregulates BAT thermogenesis, while ERK and NF-κB pathways downregulate BAT thermogenesis. Studies with kinase inhibitors showed that the inhibition of p38 MAPK significantly reduced the UCP1 expression in brown adipocytes treated with TNFα, whereas the inhibition of ERK showed an opposite consequence. Thus, it's likely that the overall effect of TNFα on BAT thermogenesis depends on the balance of all the pathways that are involved in the regulation of UCP1 expression and thermogenesis.

## 2.3 Endotoxin (LPS) in obesity

In addition to the hypertrophic adipose tissue oriented inflammation, recent studies have discovered that the alteration of gut microbiota population is a new mechanism that connects high fat diet and insulin resistance. Studies have demonstrated that high fat diet (HFD) increased the proportion of lipopolysaccharide (LPS) containing microbiota in the gut and increased intestinal permeability, which allows LPS to get into circulation. As a consequence, the plasma levels of LPS are mildly increased, which is hypothesized to mediate HFD-induced metabolic disturbance [96, 97]. Emerging evidence has indicated that LPS binds to the toll-like receptor (TLR) 2, 4 and has a profound impact on white adipose tissue biology, including inducing inflammation, disrupting lipid metabolism and promoting insulin resistance [98-100].

The two main downstream pathways of TLR2/4 have been well studied in immune cells, and some studies have shown the similar regulation in mature adipocytes [101]. First, the activation of TLR2 and 4 turns on the TIRAP/MyD88 dependent pathway. This triggers a signaling cascade and ultimately activates of IKK$\beta$ and MAPKs such as p38 MAPK and extracellular signal-regulated kinase (ERK) 1/2. These kinases in turn activate the transcription factor NF-κB and the production of pro-inflammatory cytokines and chemokines such as TNFα and IL-6. In another MyD88-independent pathway, TLR4 can activate TRAM/TRIF. These adaptors in turn activate interferon regulatory factor-3 (IRF3), which induces type I interferon (IFN) expression and involves the late phase of NF-κB activation [102].

The mechanisms of LPS to induce insulin resistance in adipose tissue have been intensively studied in several models, such as 3T3-L1 cells and primary human/mouse adipocytes. Beyond the production and secretion of pro-inflammatory cytokines, the activation of TLRs in adipocytes can impair insulin signaling via activating MAPK, which stimulates serine phosphorylation of insulin receptor substrate 1 [103]. In addition, the activation of TLRs induces lipolysis, which leads to dyslipidemia and systemic insulin resistance [99]. Moreover, several studies have demonstrated that LPS impairs adipogenesis. For example, the exposure to LPS during adipocyte differentiation led to the inhibition of adipocyte differentiation of 3T3-L1 cells and human progenitor cells [104, 105]. This effect of LPS further decreases lipid storage capacity in adipose tissue, contributing to the development of dyslipidemia in HFD-induced obesity.

### 2.4 Inflammation inhibits the browning of white adipose tissue

Different from the above-mentioned contradictory role of inflammation in BAT thermogenesis, quite a few of studies demonstrated that blocking inflammation promotes the browning of white adipose tissue in various rodent models. Studies with group 2 innate lymphoid cells [106], administration of type 2 cytokines IL-4 [57], overexpression of VEGF [107], and knockdown of RIP140 in macrophage [108] have suggested that reducing pro-inflammatory M1 macrophage polarization or alternatively increasing M2 macrophage activation could be an important approach to induce the browning of adipose tissue. Additionally, amelioration of adipose tissue inflammation by other means, such as genetic inhibition of serotonin synthesis in adipose tissue [109], suppression of interferon

signaling [110] and knockout of response gene to complement 32 [111] all could induce the browning of adipose tissue. In most of these studies, corresponding improvement of mitochondrial activity, increase of energy expenditure and reduced weight gain were observed under the high fat diet condition, which ultimately led to the improvement of insulin sensitivity.

## 3. Mitochondrial function and insulin sensitivity

As mentioned previously, fatty acid is not toxic when stores in lipid droplets as triglyceride. However, as a consequence of positive energy balance, substantial amount of lipids come to adipose tissue and adipocytes expand to store them. When adipocyte expansion reaches its limited capacity, lipolysis will be elevated and fatty acids will be released into circulation, which leads to lipotoxicity and insulin resistance. Since fatty acid is primarily a form of energy, the elevation of fatty acid oxidation in mitochondrion and energy expenditure is theoretically beneficial to insulin resistance. This notion has been proved by studies looking at the "browning" of WAT, which is speculated to substantially increase energy oxidation in adipose tissue. Since the mitochondrial function in adipocytes has been valued critical in regulating energy balance and insulin sensitivity, I summarize the biology and pathology of mitochondrion in adipocyte.

### 3.1 Mitochondrial function in oxidative metabolism

Mitochondrion plays a key role in energy metabolism as a "powerhouse unit" of the cell. Many key metabolic reactions take place in mitochondrion such as pyruvate oxidation, fatty acid β-oxidation, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (OXPHOS) [112].

Eukaryotic cells have the ability to alter mitochondrial biogenesis. In order to adapt energy demands during growth or stress, cells change the morphology or remodel the organization and distribution of mitochondria [113]. PGC1α plays a central role in the regulation of mitochondrial biogenesis and oxidative function. The expression of PGC1α is regulated by the nutrition status; increased NAD+/NADH and AMP/ATP ratios activate PGC1a via SIRT1-mediated deacetylation and/or AMPK-mediated phosphorylation, respectively[114, 115]. PGC1α regulates the activation of multiple transcriptional factors including the nuclear respiratory factors, NRF-1 and NRF-2, and members of the nuclear receptor (NR) superfamily such as the peroxisome proliferator-activated receptor α (PPARα) and estrogen-related receptors or ERRs (ERRα, ERRβ, and ERRγ). Activation of these transcription factors is critical for the control of mitochondrial biogenesis and oxidative metabolism including fatty acid β-oxidation, the TCA cycle, mtDNA replication and OXPHOS [116]. In addition, SIRT3 has been shown to activate PGC-1α, which is required for the induction of several components of the ROS-detoxifying machinery [117].

### 3.2 Mitochondrial dysfunction and insulin resistance

Mitochondrial deficiency has been linked to the major classes of age-related disease, including diabetes, neurodegenerative disease, cardiac disease, and cancer [118-120]. The impaired mitochondrial function has been found in muscle, liver, adipose tissue, and beta cell in insulin resistant individuals, which is indicated by reduced insulin-stimulated glucose uptake and/or metabolism as well as reduced mitochondrial OXPHOS activity [121].

In adipocytes, normal mitochondrial function has been proved to be critical for the regulation of insulin sensitivity. Mitochondrial dysfunction is closely associated with insulin resistance. Although HFD could temporarily increase mitochondrial number and activity due to a prompt adaptive response, a chronic increase in fuel influx can induce overproduction of reactive oxygen species (ROS) and mitochondrial damage [122, 123]. Downregulation of PGC1α in caloric excess state could be the main cause of oxidative stress resulting from mitochondrial dysfunction and ROS overproduction. The dysfunctional mitochondria lead to the profound changes in adipocyte metabolism and function, including the inhibition of pre-adipocyte differentiation, increased lipid peroxidation, decreased fatty acid β-oxidation and decreased OXPHOS activity. All of these changes contribute to the development of insulin resistance [124].

### 3.3 Reactive oxygen species (ROS)

Mitochondrial electron transport chain (ETC) delivers electron pairs to molecular oxygen, the terminal acceptor, to form H2O. In this process, molecular oxygen

occasionally becomes partially reduced by a single electron at complexes I and III, and forms superoxide anion ($O2^{\cdot -}$), which belongs to the toxic reactive oxygen species (ROS). Moderate generation of ROS in adipocytes during insulin stimulation enhances insulin sensitivity via stimulating insulin receptor autophosphorylation and PTEN oxidation, leading to the activation of downstream signaling events through the PI3K/Akt cascade [125, 126]. However, under the condition of overnutrition, the long-term exposure to high levels of ROS alters mitochondrial function, thereby causing insulin resistance.

There are two theories regarding how ROS induces insulin resistance. One theory explains that accumulation of ROS leads to nonspecific oxidative damage to mitochondria. The other is that mitochondrial ROS are part of a redox signaling pathway that modulates the balance between energy utilization and storage [127]. Disruption of these redox signals by chronic overnutrition and lack of ATP demand contribute to the development of metabolic syndrome phenotype. Since the levels of oxidative damage to mitochondria and the changes in the specific activity of components of mitochondrial ETC complexes tend to be relatively modest at least at the beginning state of metabolic syndrome [128], the second theory seems to be more favorable for explaining the initiation of metabolic dysregulation.

Within the redox signaling pathway, aconitase in TCA cycle is particularly sensitive to superoxide since this enzyme has an iron–sulfur center in its active site. Superoxide inactivates aconitase, leading to the accumulation of citrate in the matrix, which can be exported to cytoplasm for fatty acid synthesis [129]. Therefore, ROS leads to the diversion of acetyl-CoA away from oxidative phosphorylation and towards

lipogenesis. Another form of ROS is hydrogen peroxide, which can be converted from superoxide by superoxide dismutase. Hydrogen peroxide easily diffuses from the mitochondrial matrix to the cytosol, and thereby acts as a signal to the rest of the cell [130]. It inhibits pyruvate dehydrogenase kinase 2 (PDHK2), which in turn activates pyruvate dehydrogenase complex (PDC), facilitating the movement of carbohydrate to acetyl-CoA and into the TCA cycle. Coupled with the inactivation of aconitase by superoxide, these signals enable mitochondria to respond to overnutrition by slowing down the TCA cycle and fatty acids oxidation, while simultaneously increasing the breakdown of carbohydrates for lipogenesis. In addition, hydrogen peroxide acts to modify the function of protein involved in the breakdown of fatty acids [127]. Defects in mitochondrial fatty acid oxidation increase intracellular fatty acid intermediate metabolites (fatty-acyl-CoA and diacylglyerol), which activate stress-sensitive kinases such as PKC, IKK and JNK and in turn disrupt insulin signaling.

Moreover, insulin signal regulates mitochondrial DNA and protein synthesis, and potently stimulates mitochondrial oxidative capacity and ATP production. Therefore, the impaired insulin signaling pathway in the insulin resistant state further deregulates mitochondrial biogenesis and function.

In summary, positive energy balance is believed to be the fundamental cause of obesity and adipose tissue expansion; inflammation in hypertrophic adipose tissue is a critical inducer of adipose tissue dysfunction, which ultimately leads to systemic insulin resistance and type 2 diabetes. Elevation of LPS induced by high fat diet is a recently

identified mechanism of adipose tissue inflammation. Therefore, it's of interest to expand our understanding of LPS effect on adipocytes and to identify new molecular players that involve in this signaling pathway. In the meantime, exploring effective nutritional strategy to regulate energy metabolism and prevent insulin resistance is equally important.

# CHAPTER 2

# EFFECT OF LIPOPOLYSACCHARIDES ON ADIPOGENIC POTENTIAL AND PRE-MATURE SENESCENCE OF ADIPOCYTE PROGENITORS

The chapter is modified from the manuscript:

Ming Zhao and Xiaoli Chen. Effect of lipopolysaccharides on adipogenic potential and pre-mature senescence of adipocyte progenitors. American Journal of Physiology: Endocrinology and Metabolism. *Under review* (2nd minor revision).

## SUMMARY

The elevation of circulating LPS has been associated with obesity and aging. However, whether and how LPS contributes to adipose tissue dysfunction is unclear. In this study, we investigated the effect of LPS on the adipogenic capacity and cellular senescence of adipocyte progenitors. Stromal-vascular cells were isolated from inguinal adipose tissue of C57BL/6 mice and treated with LPS during the different time periods of adipocyte differentiation. We found that LPS treatment for 24hr prior to the induction of differentiation led to the most profound effect on the inhibition of adipogenesis, as evidenced by the morphological changes and the decreased mRNA expression of adipocyte marker genes. In addition, LPS induced features of pre-mature senescence of SV cells, including the activation of p53, the elevation of SA-β-gal activity and increased hydrogen peroxide production, but not telomere length. Upon LPS treatment, SV cells also developed senescence-associated secretory phenotype (SASP), as demonstrated by the increased expression of TNFα, IL-1β, IL-6, MCP1 and VEGFα. Blocking LPS-induced NF-κB activation and cytokine production by Bay 11-7082 failed to rescue the impaired adipogenesis and the reduction in PPARγ and Zfp423 expression. On the contrary, *Rosiglitazone* had little effect on cytokine production, but corrected the defective adipogenic potential. In conclusion, we demonstrate that LPS inhibits adipogenesis by disrupting the differentiation of adipocyte progenitors in a NF-κB-independent manner; LPS also induces pre-mature senescence of adipocyte progenitors. Our data suggest that LPS could be a potential contributor to the defective adipogenesis and the development of cellular senescence in adipose tissue during obesity and aging.

# INTRODUCTION

The main functions of white adipose tissue (WAT) are to store lipids and secrete metabolic regulators of adipokines and cytokines. Dysfunctional adipose tissue, characterized by reduced adipogenesis and increased cellular senescence and inflammation, commonly associates with obesity and aging [131]. Adipose tissue dysfunction impairs lipid storage and dysregulates production of adipokines and cytokines, leading to metabolic derangement, adipose tissue inflammation, insulin resistance, and type 2 diabetes [132-135].

Adipogenesis relies on the formation of new adipocytes from adipocyte progenitor cells. Adequate adipogenesis capacity is essential to sequester lipids in adipose tissue, which protects against the ectopic fat deposition and the development of insulin resistance [136-138]. Adipocyte hyperplasia has been considered as a protective mechanism when compared with adipocyte hypertrophy in terms of the metabolic consequences of adipose tissue expansion [139]. In diet-induced obesity, adipocyte hyperplasia (adipogenesis) is impaired. As a result, adipocyte hypertrophy and insulin resistance are developed. Studies in lean and obese subjects have shown that in obese adipose tissue, the number of adipocyte progenitor cells is increased by up to 10 fold, while the mature adipocyte number is decreased compared to lean adipose tissue [51, 131]. This observation suggests that there is an imbalance between the formation of new adipocytes and the death of mature adipocytes in obese adipose tissue, leading to a reduction in lipid buffering capacity in adipose tissue. As a consequence, excessive fatty acids are released into the circulation, contributing to the development of ectopic fat

accumulation, inflammation, and insulin resistance [140]. Studies also indicate that the decreased total adipocyte number remained several months after the removal of high fat diet (HFD), indicating that HFD has a long-term impact on the disruption of adipogenesis [51].

Cellular senescence refers to the irreversible arrest of cell cycle progression [141] and it can be categorized into two different types, i.e. replicative senescence and stress-induced senescence. Replicative senescence can occur after a certain number of mitotic divisions in aged cells [142], while stress-induced senescence can be induced anytime during the exposure of cells to certain stresses [143] such as increased levels of reactive oxygen species (ROS), irradiation, and other metabolic signals and inflammatory insults [144-147]. Some common features of senescent cells include the activation of p53 and p38 MAPK signaling pathways, senescence-associated secretory phenotype (SASP) and increased β-galactosidase activity and ROS production [148-151]. Moreover, in adipocyte progenitor cells, senescence leads to decreased differentiation capacity and altered production of extracellular matrix (ECM) proteases [152, 153]. This cellular process is associated with age-related metabolic disorders. In addition, obesity has been shown to accelerate the development of cellular senescence in adipose tissue [154].

Adipogenesis can be regulated at the early stages by controlling the intrinsic differentiation potential and at the late differentiation stage by regulating the lipid accumulation process. It would be of interest to know if HFD impairs the intrinsic differentiation potential which may be associated with its long-term effect on adipogenesis. It has been well accepted that chronic low-grade inflammation in both

obese and aged subjects is an important mediator of adipose tissue dysfunction. Studies have demonstrated that high fat diet (HFD) alters intestinal microbial profile and increases intestinal permeability, leading to increased production and leaking of lipopolysaccharide (LPS) [96]. As a consequence, the plasma levels of LPS are mildly increased, which is hypothesized to mediate HFD-induced metabolic disturbance [96, 97]. In addition, LPS levels were also found to be increased in the circulation in aged humans [155]. Emerging evidence has indicated that LPS binds to the Toll-like receptor 4 and has a profound impact on white adipose tissue biology, including inducing inflammation, disrupting lipid metabolism and promoting insulin resistance [98, 99].

With regards to the regulation of adipogenesis, studies from *in vitro* cultures of 3T3-L1 cells and human adipocyte progenitor cells consistently demonstrated that LPS treatment during adipocyte differentiation led to the inhibition of adipocyte differentiation [104, 105]. Previous reports have also shown that LPS induces cellular senescence in multiple types of cells [156-158]. However, the effect of LPS on the intrinsic adipocyte differentiation potential and senescence of adipose stromal-vascular (SV) cells has not been investigated previously. It has been shown that there are fat depot differences in the regulation of metabolism, the expression of adipogenic genes such as PPARγ, Zfp423, and Pref1, and adipogenic capacity [159-161]. For instance, adipocyte progenitor cells derived from inguinal fat depot have better adipogenic potential than those from epididymal fat depot [162]. It is possible that LPS may have a differential impact on adipogenesis and metabolism in different depots. However, most of previous *in vitro* studies were conducted in 3T3-L1 cells, focusing on the effect of LPS on inflammation, lipid metabolism and adipogenesis [104, 105, 163-166]. The effect of LPS

on the intrinsic differentiation potential of adipocyte progenitors using primary inguinal SV cell cultures which reflect better the physiological conditions has not been previously reported.

In this study, we explored the impact of LPS on the intrinsic adipogenic capacity as well as the development of cellular senescence of inguinal SV cells. Additionally, we determined the role of NF-κB signaling pathway in mediating these effects of LPS. We found that LPS inhibited the intrinsic adipogenic potential of inguinal SV cells in a NF-κB-independent manner. Rosiglitazone could block the LPS inhibition of adipogenesis, but not LPS-induced inflammation in inguinal SV cells. Moreover, SV cells underwent pre-mature senescence in response to LPS treatment.

## MATERIALS AND METHODS

### *Animals*

C57BL/6 mice purchased from Jackson Laboratory were housed in specific pathogen-free facility at the University of Minnesota. Animal handling followed the U.S. National Institutes of Health guidelines, and experimental procedures were approved by the University of Minnesota Animal Care and Use Committee. Mice were maintained on a 12:12-h light-dark cycle, with food and water available ad libitum.

### *Cell culture*

Stromal-vascular (SV) cells were isolated from inguinal WAT of male C57BL/6 mice at 12-14 weeks of age as described previously [167]. Fat pads were minced and digested in Krebs-Ringer bicarbonate HEPES (KRBH) buffer (pH 7.4) with collagenase (2 mg/ml solution). After a 1-h digestion, SV cells were separated by centrifugation at 1500 rpm for 10 min and washed twice with KRBH buffer. SV cells were then collected and cultured in growth medium (DMEM plus 10% FBS) until confluence. Confluent cells were treated with LPS at different doses for 24-hour unless otherwise mentioned in the context. After LPS treatment, cells were washed with PBS for 3 times and differentiated with a differentiation cocktail (100 IU/ml penicillin-streptomycin, 10% fetal bovine serum, 115 μg/ml methylisobutylxanthine, 390ng/ml dexamethasone, and 1 μg/ml insulin) for 3 days. In the corresponding experiments, 3μM Bay 11-7082 or 1 μM Rosiglitazone was added from 3 hour prior to LPS treatment to day 3 of differentiation. The cultures were then continued with DMEM with100 IU/ml penicillin-streptomycin, 10% fetal bovine serum, and 1 μg/ml insulin for another 4 days.

***Oil-Red O Staining***

Differentiated adipocytes were washed twice with PBS and fixed with Baker's Formalin for 30 min at 4°C. Cells were then stained for 10 min in freshly diluted Oil Red O solution. Finally, cells were washed with running water and observed under the microscope.

***Western blotting analysis***

Proteins were extracted from cell samples using RIPA buffer (Sigma, St. Louis, MO, USA) containing protease inhibitors and phosphatase inhibitor cocktail. Equivalent amounts of proteins were separated on 10% SDS-PAGE gel and then transferred to nitrocellulose membranes. The membranes were probed with PPARγ, C/EBPβ, NF-κB p65, phospho-NF-κB p65 (Ser536), P53, phospho-P53 (Ser15), P38 MAPK, phosphor-P38 MAPK (Thr180/Tyr182) or β-Actin (Cell Signaling, MA, USA) antibodies according to the recommendations of the manufacturers. ECL Western Blotting Detection System (GE Healthcare BioSciences, Piscataway, NJ, USA) was used to detect antibody reactivity.

***Relative quantitative real-time RT-PCR***

Total RNA was extracted from cells using the Trizol method (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from DNase-treated RNA (3μg) using a Superscript II reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). Real time-PCR was performed using SYBR Green qPCR Master Mix (Qiagen, MD, USA) with an ABI Step One Plus real-time PCR System (Applied Biosystems, Foster City, CA, USA). Primer sequences are provided in Table 1. Results are normalized to TBP using the $\Delta\Delta C_t$ method and presented as levels of expression relative to that of controls.

***Senescence Associated-β-Galactosidase activity Assay***

Senescence associated-β-galactosidase activity was determined by using commercially available kit from Thermo Scientific (Rockford, IL, USA). According to

the manufacturer's instruction, a portion of cell lysate was mixed with β-galactosidase assay reagent and incubated for 30 minutes at 37 °C. The absorbance was determined at 405nm in a microplate reader. The readings were then normalized by the protein concentration for each sample, as determined by BCA assay.

***Senescence Associated-β-Galactosidase staining***

SV cells were seeded in 6-well plates and split when reached 80% confluence. SV cells were treated without and with LPS and passaged 4 times. β-galactosidase staining was performed using cellular senescence assay kit from EMD Millipore (Billerica, MA, USA) according to the manufacturer's instruction. Briefly, cells were fixed with fixing Solution for 10 minutes. After rinsing with PBS, cells were incubated with X-Gal solution at 37°C until blue color developed. Cells were then washed with PBS and blue stained cells were counted in 10 randomly captured fields per well under microscope (100X magnification).

***Hydrogen Peroxide Assay***

Hydrogen peroxide level in cell lysate was determined by using Amplex Red Hydrogen Peroxide Assay Kit (Invitrogen, Eugene, OR, USA). Briefly, hydrogen peroxide produced by the cells was assessed by incubating the cell lysate in a reaction mixture containing Amplex Red and then determining the absorbance at 560 nm in a microplate reader, as per the manufacturer's instructions. The readings were then normalized by the protein concentration of cells for each sample.

***Telomere length measurement***

Telomere length was measured by quantitative PCR, as described previously [168, 169]. The total DNA was extracted from cultured SV cells. Samples were digested in lysis buffer (0.01 M Tris–HCl, 0.25 mM EDTA, 0.5% SDS, pH 8.3) containing 100 μg/ml proteinase K and extracted with chloroform and 8 M potassium acetate. The supernatant was collected and precipitated with isopropyl alcohol and ethanol. Telomere length was determined by detecting terminal restriction fragment (TRF) length using real-time PCR. The value was normalized to the levels of single copy gene 36B4, which encodes acidic ribosomal phosphoprotein PO.

***Statistical analysis***

Results are expressed as means ± SEM. Differences in the parameters between two groups were evaluated using Student's *t*-test with a 0.05 2-sided significance level. One way ANOVA was used to compare the differences between multiple groups. A value of $P < 0.05$ was considered significant.

# RESULTS

**Short-term exposure of inguinal SV cells to LPS reduces adipocyte differentiation**

First, we examined the effect of exposure to LPS during the different time periods of the differentiation process on the inhibition of adipogenesis. Stromal vascular (SV) cells were isolated from inguinal fat depot of regular chow diet (RCD)-fed C57/B6 mice at 12-14 weeks of age. Cells were treated with 1μg/ml LPS at the different time periods as indicated in Fig. 1A, i.e. before the initiation of differentiation (day -1 to day 0),

during the early (day 0 to day 3) and late (day 5 to day 7) stage of differentiation. When added during day -1 to 0 or day 0-3, LPS significantly inhibited adipocyte differentiation, as demonstrated by the changes in morphological differentiation (Fig. 1B) and decreased expression of adipogenic (PPARγ, C/EBPα, GLUT4, LPL) and lipogenic (SREBP1c, SCD1, FASN) genes (Fig. 1C). However, when added during the late stage (day 5-7) of differentiation, LPS did not affect the morphology of differentiated adipocytes (Fig. 1B), but caused the down-regulation of mRNA expression of genes involved in lipogenesis including PPARγ, GLUT4, LPL, SREBP1c, SCD1, and FASN (Fig. 1C). Surprisingly, treatment of SV cells with LPS only for 24h prior to the addition of differentiation cocktail (day -1 to 0) led to the most profound effect on the inhibition of adipogenesis, as evidenced by the morphological changes (Fig. 1B) and the mRNA expression of adipocyte marker genes examined in the cultures on day 7 of differentiation (Fig. 1C). Since 24h prior to the induction of differentiation is the crucial period for LPS effect on adipocyte differentiation, LPS was only added at this time period in the following experiments. Since PPARγ is the master regulator of adipogenesis, we measured its expression during differentiation. Fig. 1D shows that the suppression of PPARγ by LPS in SV cells persisted during adipocyte differentiation.

**LPS induces pre-mature senescence of adipose SV cells**

Cellular senescence of adipocyte progenitors has been associated with decreased differentiation capacity. Therefore, it is of interest to examine if LPS induces cellular senescence of adipose SV cells. Our results showed that 24h LPS treatment (day -1 to day 0) significantly induced p53 phosphorylation in SV cells (Fig. 2A). Consistently, LPS treatment led to an increase in senescence-associated β-galactosidase activity (Fig. 2B)

and β-galactosidase positive cells (Fig. 2C) as well as an upregulation of C/EBPβ protein expression in SV cells (Fig. 2A). In addition, the phosphorylation of p38 MAPK and NF-κB p65 was increased (Fig. 2A), so was $H_2O_2$ production in 24h LPS-treated SV cells (Fig. 2D). Moreover, LPS-treated SV cells exhibited significant SASP, as demonstrated by a marked increase in the mRNA expression of TNFα, IL-1β, IL-6, MCP1 and VEGFα when compared with control cells (Fig. 2E). We next determined if LPS-induced senescence is telomere-dependent. To evaluate the effect of LPS on telomere length, we passaged SV cells 4 times in the presence or absence of LPS and measured telomere length every passage. As shown in Fig 3F, telomere length was not changed after passaging 3 times in both control and LPS-treated SV cells. After passaging 4 times, telomere length was significantly shortened in control and LPS-treated cells compared to unpassaged SV cells. However, LPS treatment did not seem to accelerate telomere shortening by passaging, suggesting that LPS induces senescence via a telomere-independent mechanism.

**LPS inhibition of adipogenesis is NF-κB independent**

We explored the molecular pathways that possibly mediate LPS inhibition of adipogenesis. Inflammation has been known to play a role in regulating adipocyte differentiation. We then investigated the role of cellular inflammation in mediating LPS inhibition of adipocyte differentiation. First, we determined whether LPS induces persistent cellular inflammation which may lead to the inhibition of adipocyte differentiation. As shown in Fig. 2A, LPS treatment from day -1 to 0 significantly induced the activation of NF-κB p65 in SV cells when examined on day 0. However, the activation of NF-κB by LPS was resolved quickly. One day or 7 days after the removal

of LPS, NF-κB p65 phosphorylation was not different anymore between control cells and cells with LPS treatment during day -1 to 0 (Fig. 3A). Similar results were perceived with the mRNA expression of TNFα, IL-1β and MCP1 (Fig. 3B). Only the expression of IL-6 on day1 was still significantly higher in LPS-treated cells compared with controls. These results suggest that the impact of 24h LPS treatment on inflammatory response is not persistent during adipocyte differentiation.

Since NF-κB activation can trigger the downstream pathway that directly inhibits PPARγ activity, it is likely that NF-κB mediates the LPS inhibition of early-stage preadipocyte differentiation. To test this hypothesis, we blocked NF-κB pathway by Bay 11-7082 from 3 hours prior to LPS treatment until 3 days after differentiation. Bay 11-7082 significantly reduced LPS-induced gene expression of cytokines such as TNFα, IL-1β and IL-6 (Fig. 4A) as well as NF-κB phosphorylation (Fig. 4B), suggesting the successful inhibition of NF-κB. However, neither the down-regulation of PPARγ nor the inhibition of adipogenesis was reversed by Bay 11-7082 (Fig. 4B and 4C). To determine if the NF- κB activity impacts the long-term effect of LPS on the inhibition of adipogenesis, LPS and Bay 11-7082 were added to SV cell cultures during the entire period of differentiation process (day-1 to day7). Similar to the short-term effect of LPS, Bay 11-7082 was not able to prevent LPS inhibition of adipocyte differentiation (Fig. 4D) and adipogenic gene expression (Fig. 4E). These results suggest that LPS effect on the early stage of adipogenesis is at least in part independent of NF-κB signaling pathway activation.

**LPS regulates the expression of preadipocyte differentiation genes in SV cells**

Since LPS was removed before the induction of differentiation, the possible mechanism for its anti-adipogenic effect could be due to its impairment in the intrinsic adipogenic capacity of adipocyte progenitors. We then examined the mRNA expression of key factors controlling preadipocyte differentiation including C/EBPδ and Zfp423 and preadipocyte marker Pref-1 in SV cells after 24h LPS treatment (day -1 to 0). As shown in Fig. 5A, LPS treatment from day -1 to day 0 led to a significant reduction in the expression of Zfp423, Pref-1, and PPARγ, but an increase in C/EBPδ expression. As previously reported, C/EBPδ has a pro-adipogenic, while Pref-1 an anti-adipogenic role [159, 170]. The patterns of C/EBPδ and Pref-1 expression does not match their role in adipocyte differentiation, suggesting that they may not be directly involved in LPS inhibition of adipogenesis. It seems that the down-regulation of Zfp423 is consistent with its regulatory role in adipogenesis as an upstream activator of PPARγ (Fig. 5A). Moreover, our results showed that Bay 11-7082 had no effect on LPS-induced reduction in Zfp423 expression in SV cells (Fig. 5B). Additionally, we determined if restoring PPARγ activity can reverse LPS inhibition of adipogenesis. Rosiglitazone was given from 3 hours prior to LPS treatment until 3 days after differentiation. As shown in Fig 5C, Rosiglitazone completely restored the LPS inhibition of SV cell differentiation capacity. Rosiglitazone significantly upregulated PPARγ protein expression in both control and LPS-treated cells (Fig. 5D and 5E), without affecting Zfp423 expression (Fig. 5B). However, Rosiglitazone was not able to suppress the LPS-induced upregulation of cytokine gene TNFα and IL-6, but it did significantly inhibit LPS-induced HIF1-α and VEGFα expression (Fig. 5E).

# DISCUSSION

Adequate adipogenesis is essential for sequestering fatty acids in adipose tissue, thereby preventing ectopic lipid accumulation and maintaining insulin sensitivity [136-138]. The recruitment of new adipocytes from adipose precursor cells, a process called adipocyte hyperplasia, increases lipid storage capacity and is believed to have a beneficial role in metabolic regulation [136-138]. In this study, we investigated the LPS regulation of adipogenic potential of adipocyte progenitor cells in primary SV cells isolated from mouse inguinal adipose tissue. Compared to 3T3-L1 cells, primary adipose SV cells have been known to represent better adipocyte progenitor cells for studying adipogenesis. Our results demonstrate that the short-term exposure to LPS prior to the initiation of adipocyte differentiation permanently impairs the adipogenic capacity via an NF-κB-independent mechanism and induces telomere-independent pre-mature senescence in SV cells. These results suggest that the elevation of plasma LPS may contribute in part to the impaired adipogenesis and cellular aging of adipocyte progenitor cells in obesity and aging.

Most of previous studies conducted in adipose cell lines have focused on the inhibitory effect of inflammation on adipogenesis by examining the effect of adding inflammatory factors to the adipocyte cultures either during the entire period of differentiation process or the short-term effect on mature adipocytes [104, 171, 172]. The present study attempted to more precisely understand the target points where LPS does its inhibitory effect on adipocyte differentiation in inguinal SV cell cultures. We therefore examined the inhibitory effect of LPS by adding it to the SV cell cultures during various time periods of differentiation process. We found that LPS has its most profound

inhibitory effect when added to the SV cells 24h prior to the initiation of differentiation. This effect could not be reversed by inhibiting inflammation or blocking NF-κB activity as will be discussed further later. Our findings suggest that LPS inhibits adipogenesis via blocking the early stage of SV cell differentiation. These findings also suggest that the increased levels of endotoxin in obesity and aging could contribute to decreased adipogenesis and lipid storage capacity of subcutaneous adipose tissue. When added to differentiated adipocytes during day5-7, LPS does not affect adipocyte morphology, but disrupts adipocyte lipid metabolism. For instance, LPS significantly inhibits mRNA expression of genes involved in lipogenesis such as PPARγ, GLUT4, LPL, SREBP1c, FASN and SCD1. PPARγ has two major functions in 1) regulating adipogenesis (adipocyte differentiation) and 2) lipid metabolism in mature adipocytes. LPS treatment from day -1 to 0 affects adipocyte differentiation, while adding LPS from day 5-7 mostly affects PPARγ-mediated lipid metabolism.

Cellular senescence, a process of growth arrest, is a cellular mechanism protecting against unrestricted growth of damaged cells [173]. This event can be induced in aged subjects and in cultured cells after serial passaging [142]. The above mentioned senescence inducers activate p53, $p16^{Ink4a}$, or both, which in turn induce cell-cycle arrest [174]. Telomere shortening, which has been linked with the cellular aging process, is an inducer of replicative senescence [175], while stress-induced pre-mature senescence is mostly via a telomere-independent mechanism [143]. Studies have shown that preadipocytes isolated from aged subjects display cellular senescence, including impaired preadipocyte differentiation, increased inflammation, upregulation of C/EBPβ, and elevated senescence associated β-gal activity [131]. Moreover, preadipocytes isolated

from obese individuals show similar senescent features, indicating that obesity can induce the development of cellular senescence in adipose tissue [131]. However, the underlying factors that induce cellular senescence in obesity remain unknown. The results from a recent study [176] have shown that acute LPS injection induces the gene expression of cytokines while reducing adipogenic gene expression in visceral adipose tissue. They further demonstrated that the LPS-induced change in adipogenic and cytokine gene expression occurs primarily in the SV fraction, suggesting that SV cells could be a potential target of the LPS effect on senescence and adipogenesis *in vivo*. In this study, we showed that LPS treatment resulted in an upregulation of P53 phosphorylation and C/EBPβ protein expression induced SASP (TNFα, IL-1β, IL-6, MCP1 and VEGFα) and increased SA β-galactosidase activity in inguinal SV cells. However, LPS does not seem to accelerate telomere shortening induced by passaging cells. These results suggest that LPS serves as a potential inducer of telomere-independent pre-mature senescence of adipocyte progenitor cells. In addition, LPS stimulated HIF1-α gene expression, which has a role in the regulation of adipose tissue fibrosis [177] that commonly develops during obesity and aging. Thus, our results suggest that the elevation of LPS may also contribute to the remodeling of adipose tissue in obesity and aging. Increased C/EBPβ expression by LPS seems to be contradictory to its role in adipogenesis. However, it has been known that there are two isoforms of C/EBPβ, LAP and LIP. LAP plays a role as a stimulator of adipogenesis, while the short isoform, LIP, inhibits adipogenesis [178]. In the senescent adipose tissue, both LAP and LIP are increased, but LIP increased more significantly, which leads to an overall inhibition of adipogenesis [179]. In addition, C/EBPβ is not the only transcription factor that controls adipogenesis. Adipogenesis is

regulated by multiple transcription factors including PPARγ, C/EBPα, and C/EBPβ. Among them, PPARγ and C/EBPα are known to play key roles in controlling adipogenesis. We observed that both PPARγ and C/EBPα were decreased by LPS. Thus, we believe that decreased PPARγ and C/EBPα are likely the major contributors to the LPS inhibition of adipogenesis.

The inhibitory effects of inflammatory inducers such as LPS, TNFα and macrophage-conditioned medium on adipocyte differentiation when they are added during the adipocyte differentiation process have been extensively studied [104, 171, 172]. The mechanism for the effect of proinflammatory factors on adipogenesis is largely speculated to be through the direct activation of NF-κB pathway, which in turn reduces PPARγ activity during adipocyte differentiation process [104, 171, 172]. Therefore, we determined the role of NF-κB signaling pathway in the LPS-induced intrinsic inhibition of adipogenesis. We evaluated the dynamic change of NF-κB signaling activation as well as the effect of blocking NF-κB activity during adipocyte differentiation after LPS treatment. Interestingly, we found that the LPS-induced NF-κB activation and inflammatory cytokine production in SV cells was quickly resolved within 24h after the removal of LPS from the cultures. However, the 24h exposure to LPS prior to the addition of differentiation cocktail (from day -1 to day 0) had a permanent impact on adipogenesis and PPARγ expression even after LPS was removed from the cultures. This suggests that NF-κB signaling activation is not the direct contributor to the LPS-induced inhibition of PPARγ transcription and adipogenesis. To further confirm this conclusion, we examined the rescue effect of NF-κB inhibitor on LPS inhibition of adipogenesis. As our results shown, NF-κB inhibitor (Bay 11-7082) failed to reverse the inhibition of

adipogenesis by LPS in SV cells. Wang et al conducted similar studies in 3T3-L1 cells [104] and their results are not completely consistent with our findings. This discrepancy could be due to the differences in cell models and NF-κB inhibitors used in two groups. Wang et al used IKKβ inhibitor sc-514 instead of Bay 11-7082 to block NF-κB signaling activation in 3T3-L1 adipocytes. Most importantly, they observed that sc-514 only partially rescues LPS inhibition of adipogenesis. The differentiation rate was still 40-50% lower in cells treated with LPS plus sc-514 compared with control cells or cells treated with sc-514 alone. Thus, we conclude that LPS inhibits adipogenesis in part via a NF-κB independent mechanism.

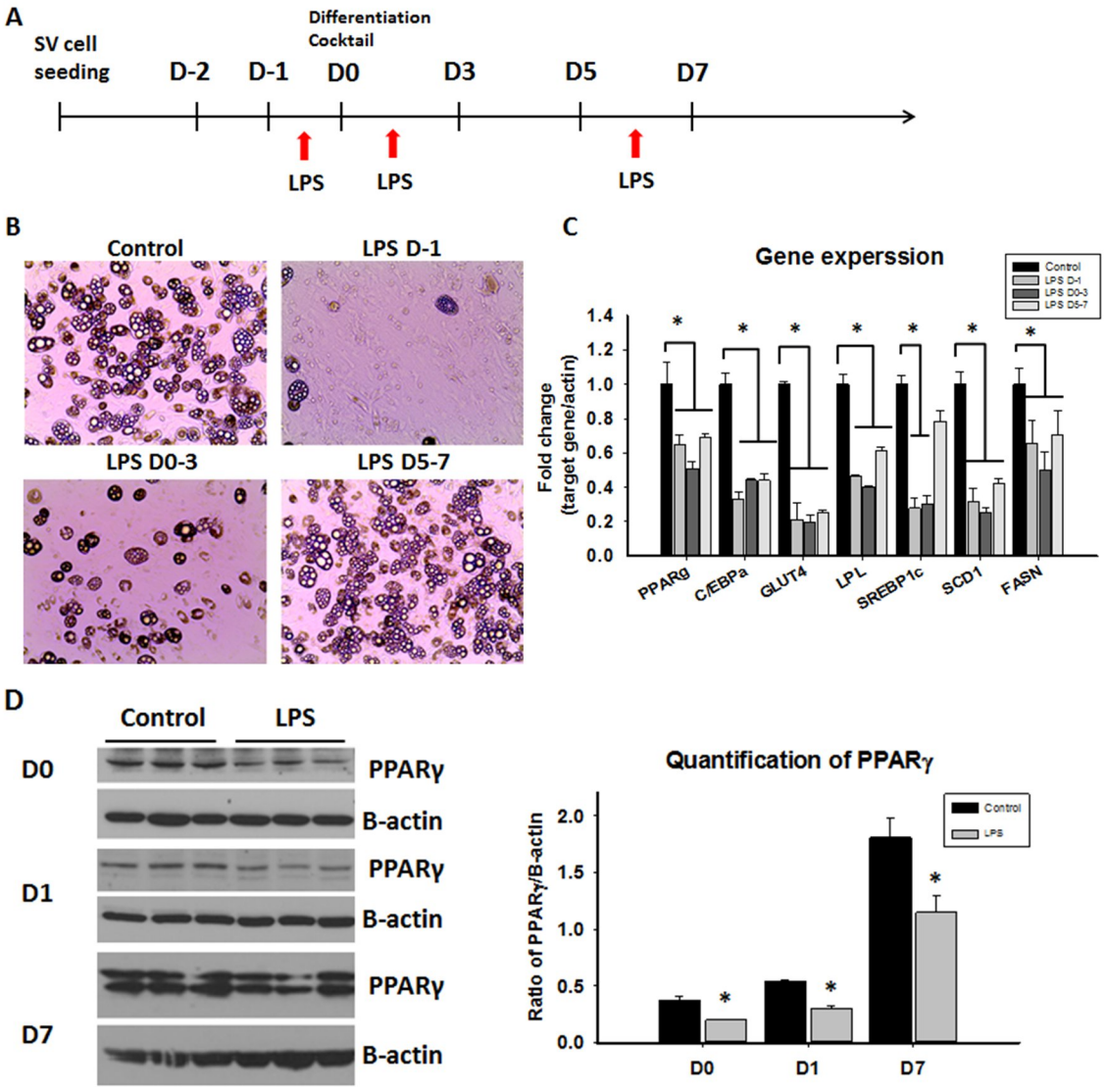
Zfp423 has recently been determined as a key regulator of adipogenesis. In a previous study, Zfp423 positive cells isolated from SV fraction of both subcutaneous and visceral adipose tissue of mice were able to stably commit to preadipocytes and differentiate into adipocytes [161]. Ectopic expression of Zfp423 in non-adipogenic NIH 3T3 fibroblasts robustly promoted adipocyte differentiation [36]. In contrast, transcriptional repression of Zfp423 by Zfp521 inhibited the commitment of mesenchymal stem cells to adipocyte lineage [180]. In the present study, we showed that the expression of Zfp423 gene was significantly down-regulated upon LPS treatment in SV cells, and the downregulation of Zfp423 was not reversed by NF-κB inhibitor. These results suggest that Zfp423 could be the effector of LPS, leading to the inhibition of intrinsic adipogenic potential. A line of evidence from in vitro studies indicates that Zfp423 is an upstream transcriptional factor that enhances PPARγ expression, thereby controlling adipogenesis [36]. Our data consistently showed that LPS suppressed both Zfp423 and PPARγ expression simultaneously. In the meantime, PPARγ agonist

rosiglitazone was able to activate PPARγ and restore adipogenesis bypassing the activation of Zfp423 expression. Moreover, previous studies have shown that knocking down Zfp423 in 3T3-L1 preadipocytes led to the upregulation of C/EBPβ expression, but the downregulation of PPARγ expression [36]. We observed the similar changes in C/EBPβ and PPARγ expression in LPS treated SV cells in this study. All these data together led us to speculate that LPS inhibition of intrinsic adipogenic potential of adipocyte progenitor cells could be attributed to the suppression of Zfp423 expression. To confirm this speculation, further studies are needed to determine whether the constitutive overexpression of Zfp423 could protect against LPS inhibition of preadipocyte commitment and differentiation.

In summary, the present study has added new knowledge of the differential role of LPS in adipocyte inflammation and differentiation. The effect of LPS in SV cells shown in this study indicates that LPS inhibits adipogenesis by disrupting the adipocyte progenitor cell differentiation which is independent of LPS stimulation of NF-κB signaling pathway and cytokine production. Additionally, LPS induces pre-mature senescence in a telomere-independent manner in adipocyte progenitors. Since plasma LPS levels are increased during HFD feeding and aging, our data suggest that LPS could be a potential contributor to the defective adipogenesis and the development of cellular senescence in adipose tissue during obesity and aging.

**FIGURES**

## Figure 1. Effect of short-term LPS treatment on adipocyte differentiation in primary inguinal SV cell cultures.



# Figure 2. Effect of LPS treatment on cellular senescence in

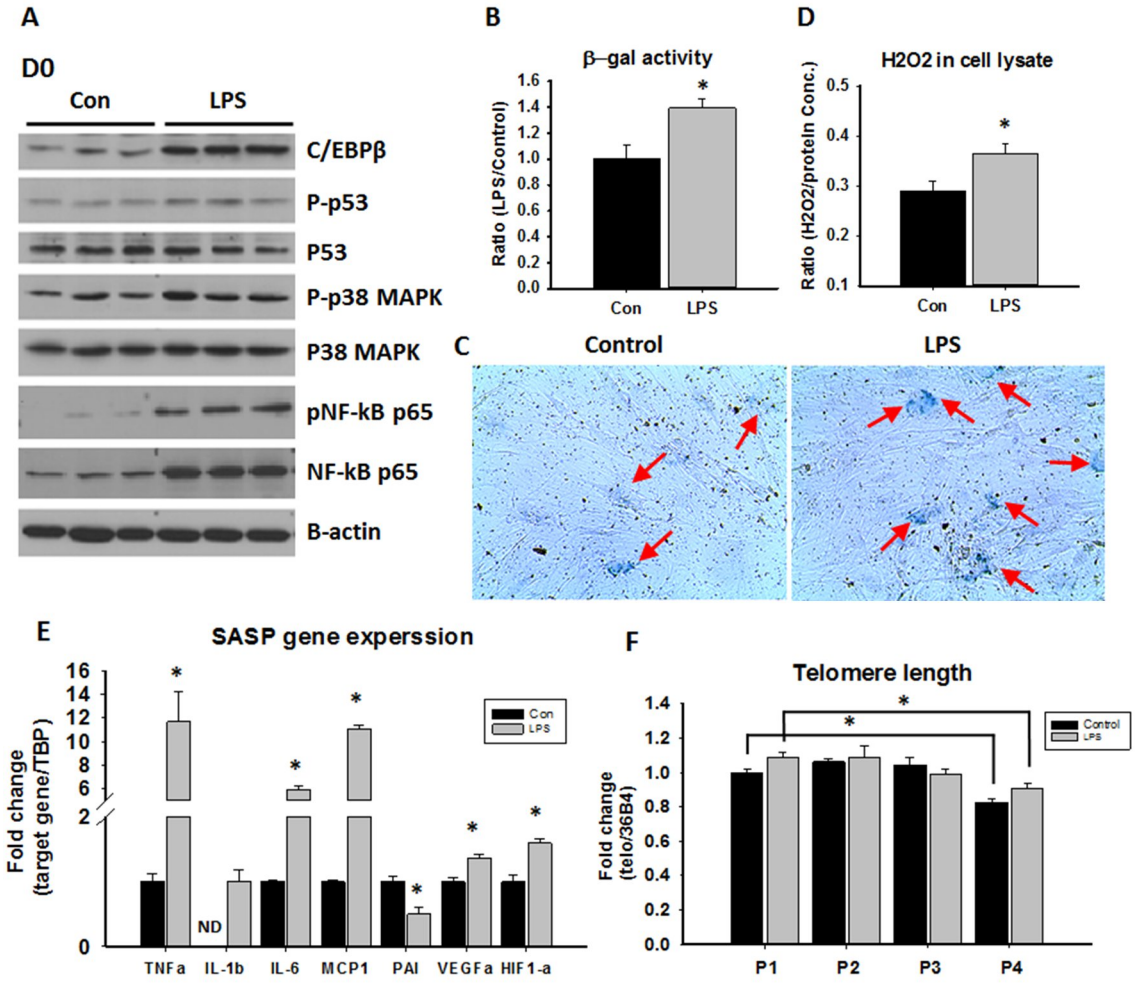


**Figure 3. Effect of LPS withdrawal on inflammatory response in primary inguinal SV cell cultures.**

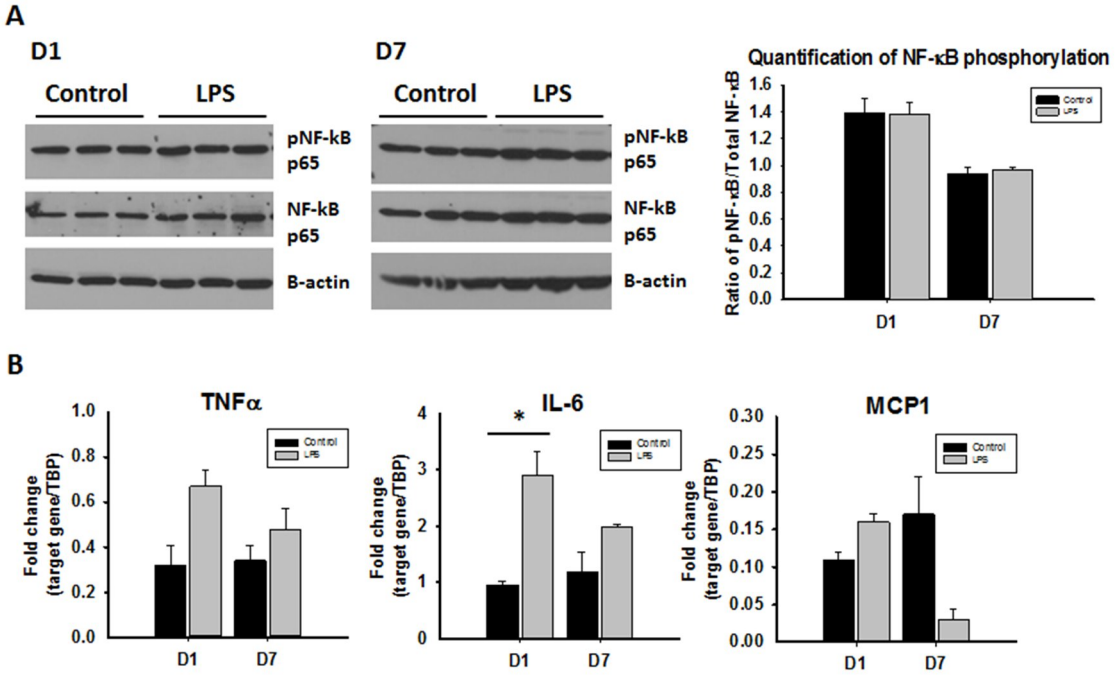


**Figure 4. Effect of blocking NF- κB activation on LPS-inhibition of adipocyte differentiation.**

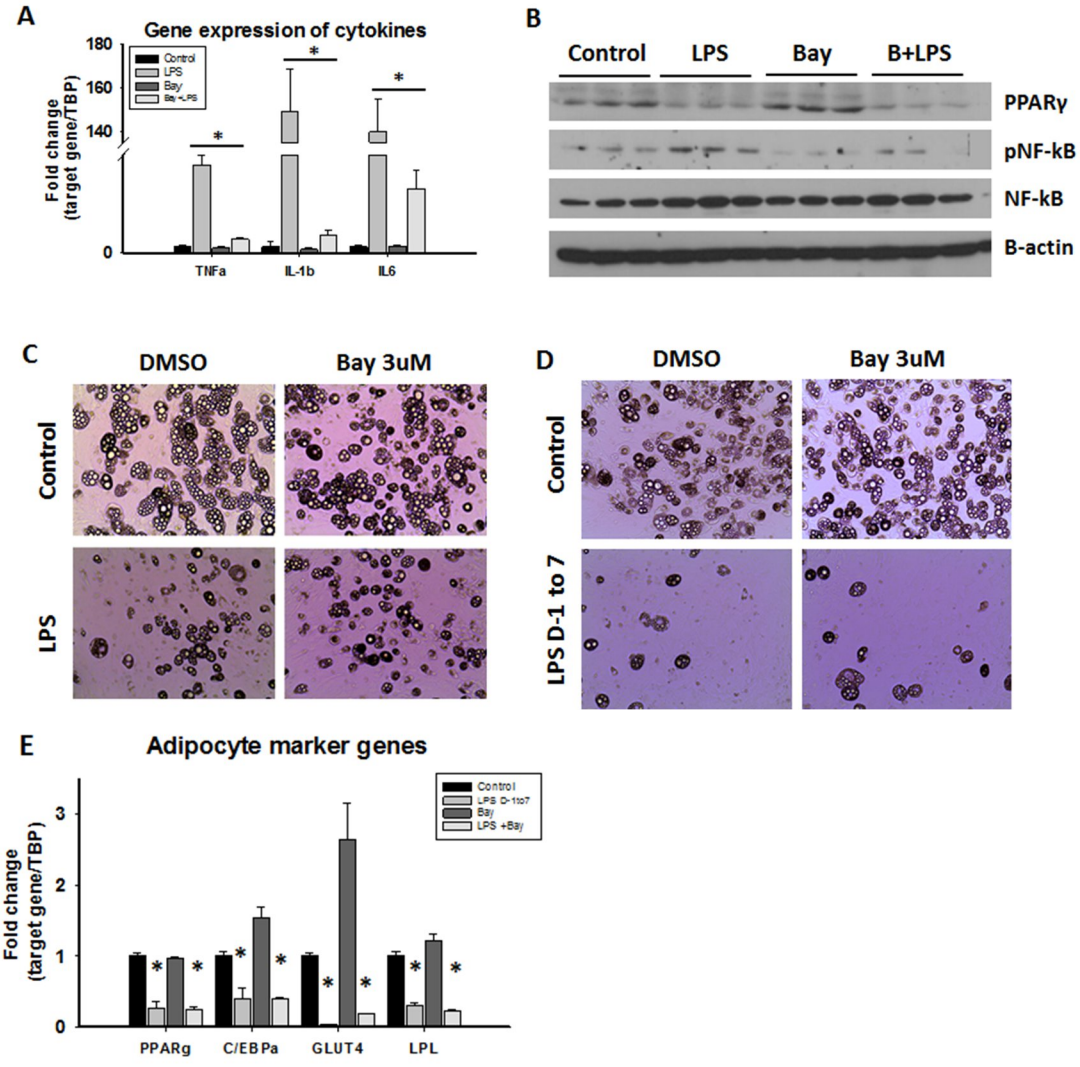
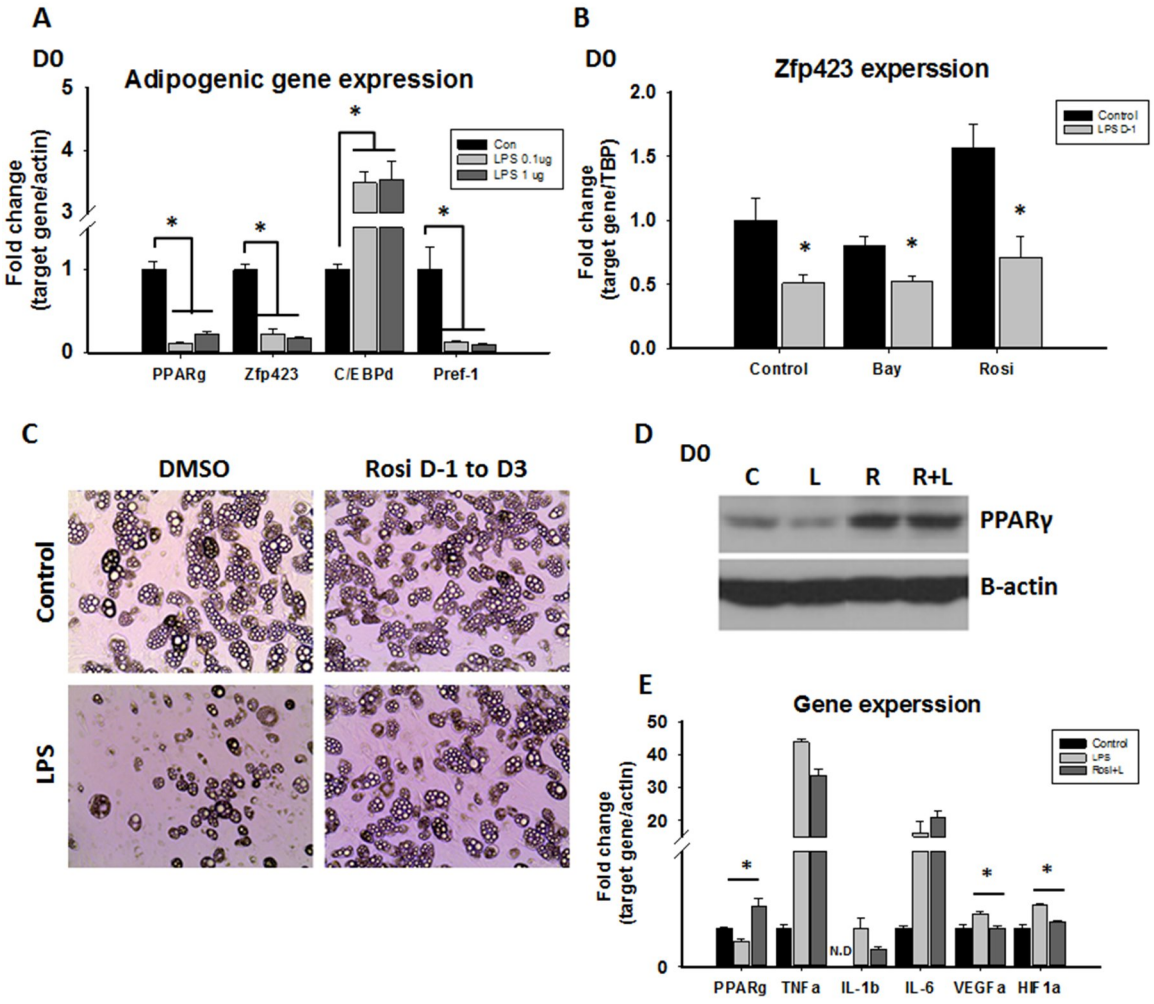


**Figure 5. Effect of LPS treatment on the expression of preadipocyte differentiation genes in SV cells.**



## FIGURE LEGEND

**Figure 1. Effect of short-term LPS treatment on adipocyte differentiation in primary inguinal SV cell cultures.** A: Schematic diagram of experimental design. B: Morphology (100 X magnification) of day 7 differentiated inguinal adipocytes with 1μg/ml LPS treatment during different periods of differentiation process. C: mRNA expression of adipocyte marker and lipogenic genes in differentiated inguinal adipocytes with 1μg/ml LPS treatment during different periods of differentiation process. D: Dynamic change of PPARγ expression in day 0, day 1 and day7 differentiated inguinal adipocytes with 24h treatment of 0.2μg/ml LPS during day -1 to day 0. In each independent experiment, SV cells isolated from 3-4 mice were pooled and cultured in 6-well plates. The 3-4 wells of cells were included for each treatment group. The experiment was repeated 2-3 times with different sets of mice. The data are represented as mean ± SEM. * $p<0.05$, compared with control.

**Figure 2. Effect of LPS treatment on cellular senescence in inguinal SV cells.** Confluent inguinal SV cells were treated with 0.2μg/ml LPS for 24 hours and subjected to the measurements of cellular senescence markers (A), β-gal activity (B) and staining (C). Production of hydrogen peroxide (D), expression of senescence featured genes (E) and telomere length (D). For telomere length and SA β-gal staining (C and F), 0.2μg/ml LPS was added to the cultures during serial passaging. The β-gal positive cells are indicated by red arrows (100 X magnification). 3-4 wells of cells were included for each

treatment group. The experiment was repeated 2-3 times with different sets of mice. The data are represented as mean ± SEM. * $p<0.05$, compared with control.

**Figure 3. Effect of LPS withdrawal on inflammatory response in primary inguinal SV cell cultures.** Confluent inguinal SV cells were treated with 0.2μg/ml LPS for 24 hours prior to adding differentiation cocktails (day -1 to day 0). After 24h treatment, LPS was removed and cocktails were added to induce the differentiation. NF-κB activity (A) and gene expression of cytokines (B) were determined at day 1 and day 7 of differentiation. The levels of gene expression in panel B are presented as fold change relative to the levels in control cells on day 0 as shown in Fig 3E. The 3-4 wells of cells were included for each treatment group. The experiment was repeated 2-3 times with different sets of mice. The data are represented as mean ± SEM. (n=3 per group) * $p<0.05$.

**Figure 4. Effect of blocking NF- κB activation on LPS-inhibition of adipocyte differentiation**. Confluent inguinal SV cells were treated with 0.2μg/ml LPS for 24 hours prior to adding differentiation cocktails (day -1 to day 0). After 24h treatment, LPS was removed and cocktails were added to induce the differentiation. Bay 11-7082 at the dose of 3μM was added from 3 hours prior to LPS treatment to day 3 of differentiation. Gene expression of cytokines (A), protein expression of PPARγ and NF-κB p65 (B) were detected in inguinal SV cell cultures 24 hours after LPS treatment. Morphology (100 X

magnification) of SV cell differentiation was determined on day 7 of differentiation(C). D and E: LPS and Bay 11-7082 were added during the entire period of differentiation process (day -1 to day 7). Morphology (100 X magnification) of SV cell differentiation (D) and expression of adipocyte marker genes (E) were determined at day 7 of differentiation. The 3-4 wells of cells were included for each treatment group. The experiment was repeated 2-3 times with different sets of mice. The data are represented as mean ± SEM. (n=3 per group) * $p<0.05$, compared with control.

**Figure 5. Effect of LPS treatment on the expression of preadipocyte differentiation genes in SV cells.** Expression of preadipocyte differentiation genes (A) in SV cells upon LPS treatment for 24 hours. 3μM Bay 11-7082 or 1 μM Rosiglitazone was added from 3 hour prior to 24-hour LPS treatment to day 3 of differentiation. The expression of Zfp423 (B) in SV cells was measured after 24-hour LPS treatment. Morphology (100 X magnification) of differentiated inguinal adipocytes (C) with or without LPS in the presence or absence of Rosiglitazone treatment were determined at day 7 of differentiation. Rescue effect of Rosiglitazone on the expression of PPARγ (D) and senescence related genes (E) in inguinal SV cells treated with LPS for 24 hours. The 3-4 wells of cells were included for each treatment group. The experiment was repeated 2-3 times with different sets of mice. The data are represented as mean ± SEM. (n=3 per group) * $p<0.05$, compared with control.

# CHAPTER 3

# KNOCKING DOWN NPC2 EXPRESSION REDUCES LYSOSOMAL ACTIVITY AND IMPAIRS INFLAMMATORY EFFECT OF LIPOPOLYSACCHARIDE IN ADIPOCYTES

The manuscript authored by Ming Zhao, Hong Guo, and Xiaoli Chen is to be submitted.

Ming Zhao performed the experiments (Fig1A-G; 2A, C, D; 3B, C, E; 4A-F; 5A, B, D) and wrote this chapter

## SUMMARY

Lysosomal activity plays an important role in controlling inflammation. Niemann-Pick disease, Type C2 (NPC2), an endosomal/lysosomal protein, is known to play an important role in intracellular transport of cholesterol. NPC2 deficiency leads to lysosomal accumulation of cholesterol. We previously reported that NPC2 expression is upregulated in adipose tissue in obesity. In this study, we investigated the role of NPC2 in regulating lysosomal activity and inflammation in 3T3-L1 adipocytes by determining the impact of NPC2 knockdown (KD) on adipocyte inflammation and function. Compared with scrambled cells, NPC2 knockdown adipocytes had similar differentiation capability. NPC2 knockdown impaired lysosomal activity in adipocytes, as evidenced by the reduced mature form of lysosomal protease cathepsin B (CtB) and decreased autophagy-lysosomal degradation leading to the accumulation of LC3 and p62. Surprisingly, NPC2 knockdown diminished LPS-induced inflammatory response including NF-κB and ERK1/2 phosphorylation and the expression of pro-inflammatory genes such as TNFα, IL18, MCP1 and MMP9. The mRNA expression of ECM genes (FN, Col1, Col3, Col4 and αSMA) was down-regulated in NPC2 KD adipocytes under the basal condition when compared with scrambled controls. Moreover, NPC2 KD blunted LPS-stimulated glucose uptake in adipocytes. The normal expression of TLR2 and 4 genes by LPS suggests that the reduced response to LPS was not due to the defects in the gene expression of TLRs. Our results suggest that NPC2 plays a role in regulating lysosomal activity and modulating LPS-induced inflammatory signaling pathway activation. Considering the similar structure of NPC2 with TLR co-activator MD-2, we

propose that NPC2 may mediate the binding of LPS to TLR4 which takes place in endosomes, thereby regulates inflammatory signaling activation.

## INTRODUCTION

Our understanding of the role of adipose tissue in metabolism has expanded in the last decade. Adipose tissue is now considered as not only a lipid storage site, but also an endocrine and immunologically active site [181, 182]. Chronic low-grade inflammation in adipose tissue has been well-established as a trigger of metabolic disorders such as insulin resistance [53, 183]. There are several models proposed for explaining the cause of adipose tissue inflammation, such as oxidative stress, ER stress, and fatty acid overload. Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, has been recently considered as a critical stimulus of adipose tissue inflammation. The data from human studies has shown that obese subjects have altered gut microflora, leading to the elevation of circulating LPS [184]. In addition, circulating LPS levels were found to be increased in aged humans [155]. Consistently, high fat diet feeding led to an increase in the proportion of LPS-containing microbiota in the gut and LPS concentration in circulation in mice [185]. LPS has been known to bind toll like receptor (TLR) 2 and 4 on the plasma membrane with the involvement of MD-2 to activate an immune response and induce inflammation [100]. Adipocytes and macrophages share many similarities in terms of their responses to LPS, since they both express TLRs, and secrete inflammatory cytokines and chemokines, such as TNFα and IL-6 [101]. In this sense, understanding the regulation of inflammation in adipocytes could be as important as that in macrophages.

Aging is a risk factor for the development of chronic metabolic diseases such as type 2 diabetes, cardiovascular disease, neurodegenerative disease, and cancer. Continuous turnover of intracellular proteins is essential for the regulation of multiple cellular functions, thereby maintaining cellular homeostasis. Cellular aging is associated with the disruption of intracellular protein homeostasis, leading to the accumulation of damaged cellular components including oxidized and misfolded proteins and damaged organelles [186, 187]. The autophagy-lysosome system is one of the main intracellular proteolytic systems that are responsible for the clearance of unwanted intracellular macromolecules and organelles [188]. Induction of Autophagy-related genes (ATG) initiates the formation of autophagosomes. During the activation of autophagy, Microtubule-associated protein 1A/1B-light chain 3-I (LC3-I) is converted to Light Chain 3-II (LC3-II) which can be recruited to the autophagosomal membrane to mediate the formation and elongation of autophagosomes [189]. In the next stage, p62/SQSTM1 recognizes polyubiquitinated protein aggregates and delivers them to the autophagosomes via the interaction with LC3-II [190]. In the final stage, autophagosomes are fused with lysosomes, and the autophagic cargo is degraded by different hydrolytic enzymes in the autophagolysosome [191]. Beyond removing damaged proteins and organelles, the autophagy-lysosome system also plays a critical role in regulating energy homeostasis, cell differentiation and defense against invading microorganisms [192]. In immune cells, it has been well documented that inflammatory signals stimulate the autophagy-lysosome activity [193-195], which can in turn clear pathogens and resolve inflammation. Although adipocytes act similar to macrophages in many ways in adipose tissue inflammation, the role of the autophagy-lysosome system in regulating inflammation in adipocytes has not

been clearly elucidated. Several lines of evidence have shown the concurrence of increased inflammatory cytokines (such as TNFα, IL-1β, IL-6, MCP1) and decreased lysosomal activity in adipose tissue during aging [149, 151, 196, 197], suggesting that the autophagy-lysosome system may play a similar role in regulating inflammation in adipocytes.

Niemann-Pick disease, Type C (NPC) proteins, which are located in the late endosome and lysosome, consist of two major members: membrane bound NPC1 and soluble NPC2. The deficiency of either of them leads to a rare autosomal recessive disease called Niemann-Pick C disease, with symptoms including neurodegeneration, hepatosplenomegaly and pre-mature death [198]. More than 95% of the cases of Niemann-Pick C disease are due to a defect in the NPC1 gene [199], while the defects in the NPC2 gene are responsible for the remaining 5% [200]. Biochemical studies have unraveled that NPC1 and NPC2 function in concert to facilitate the export of unesterified cholesterol from endosomes and lysosomes [201, 202]. Therefore, either NPC1 or NPC2 deficiency causes the accumulation of cholesterol and other cargo in the late endosomes and lysosomes, which disrupts the normal lysosomal activity. Interestingly, several recent studies unveiled that NPC2 has some NPC1-independent effects. First, NPC2 transports a small portion of endosomal cholesterol to mitochondria in Chinese hamster ovary cells, where cholesterol is converted to oxysterols and steroids to maintain mitochondrial membrane structure and function [203]. Secondly, NPC2 is secreted into plasma, bile and epididymal fluid in rodents and humans [204, 205]. However, the level of secretory NPC2 significantly varies among different mouse strains, and the biological role of secretory NPC2 has not been well elucidated. Although the accumulation of unesterified

cholesterol in the late endosome/lysosome is commonly believed to be the cause of NPC disease, the physiological role of NPC proteins, especially NPC2 has not been fully understood.

While most of previous studies on NPCs focus on their role in nervous tissue, liver and spleen, few studies have investigated the connection of NPC proteins to obesity and insulin resistance. A single-nucleotide polymorphism (SNP) in NPC1 has been associated with obesity and type 2 diabetes, and genetic inhibition of NPC1 causes insulin resistance in adipocytes [206]. Less is known for NPC2. Our lab has previously reported that high-fat diet feeding upregulates the mRNA and protein expression of NPC2 in epididymal adipose tissue in mice [207]. Consistently, others reported that NPC2 expression is upregulated in subcutaneous abdominal adipose tissue of morbidly obese women compared to non-obese subjects [208]. However, the role of NPC2 in adipocyte metabolism and function and how NPC2 deficiency contributes to obesity and obesity-related metabolic disorders remains unclear. Considering the concurrent upregulation of inflammation and NPC2 expression in obese adipose tissue, and the role of NPC2 in lipid metabolism in lysosome, we hypothesized that NPC2 may play an important role in autophagy-lysosomal function in adipocytes, thereby regulating adipocyte metabolism and inflammation. In this study, we demonstrate that NPC2 is critical for regulating lysosomal activity; and NPC2 deficiency reduced the response of adipocytes to LPS stimulation in inflammatory signaling pathway activation and glucose metabolism.

# MATERIALS AND METHODS

### *Generation of NPC2 Knockdown 3T3-L1 Cells*

The shRNA was generated with ViraPower Lentiviral Expression Systems (Invitrogen, Carlsbad, CA) by the Minnesota Obesity Center, University of Minnesota. Oligos of RNAi stem loops directed against mouse NPC2 were synthesized and cloned into a lentiviral-based RNAi vector pLKO.1. Two shRNA sequence variants for NPC2 gene were synthesized and cloned into the vector pLKO.1.Recombinant lentiviruses were generated and tested for the efficiency. The selected oligomers targeting NPC2 sequence were 5'-GCTCTCGTTCTTTGGTAGTTT-3', and 5'-CGGTTGTAAGAGTGGAATCAA-3'. Recombinant lentivirus containing a scrambled siRNA sequence was used as a control. 3T3-L1 fibroblasts at 70-80% confluence were transducted with virus supplemented with 6 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO) for 12 h. Cells were gently washed in PBS and cultured for 6 h before screening with 2 μg /ml puromycin (Sigma-Aldrich). Non transducted cells died after 7 days, and 2 colonies of survived transducted cells were picked for testing NPC2 gene expression. One colony with good knockdown efficiency and differentiation capacity was selected for experiments.

### *3T3-L1 Cell culture*

3T3-L1 cells were cultured in growth medium (DMEM plus 10% BCS) until two days after confluence. Cells were then differentiated with a differentiation cocktail (100 IU/ml penicillin-streptomycin, 10% fetal bovine serum, 115 μg/ml

methylisobutylxanthine, 390ng/ml dexamethasone, and 1 μg/ml insulin) for 2 days. The cultures were then continued with DMEM with100 IU/ml penicillin-streptomycin, 10% fetal bovine serum, and 1 μg/ml insulin for another 5 days. Full differentiated 3T3-L1 adipocytes were used for experiments. 100nM BafA1 was given for 24 hours to inhibit lysosomal activity and autophagic flux. 0.5μg/ml LPS was given for 24 hours to initiate inflammation.

***Western blotting analysis***

Proteins were extracted from cell samples using RIPA buffer (Sigma, St. Louis, MO, USA) containing protease inhibitors and phosphatase inhibitor cocktail. Equivalent amounts of proteins were separated on 8-10% SDS-PAGE gel and then transferred to nitrocellulose membranes. The membranes were probed with Cathepsin B& L, NPC2, NF-κB p65, phospho-NF-κB p65 (Ser536), ERK1/2, phosphor ERK1/2 (T202/Y204), MMP9, LC3, P62, Beclin-1, Atg7, Akt, phosphor-Akt (Ser473), or β-Actin (Cell Signaling, MA, USA) antibodies according to the recommendations of the manufacturers. ECL Western Blotting Detection System (GE Healthcare BioSciences, Piscataway, NJ, USA) was used to detect antibody reactivity.

***Relative quantitative real-time RT-PCR***

Total RNA was extracted from cells using the Trizol method (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from DNase-treated RNA (3μg) using a Superscript II reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). Real time-PCR was performed using SYBR Green

qPCR Master Mix (Qiagen, MD, USA) with an ABI Step One Plus real-time PCR System (Applied Biosystems, Foster City, CA, USA). Primer sequences are provided in Table 2. Results are normalized to TBP using the $\Delta\Delta C_t$ method and presented as levels of expression relative to that of controls.

***Oil-Red O Staining***

Differentiated adipocytes were washed twice with PBS and fixed with Baker's Formalin at 4°C for 30 min. Cells were then stained for 10 min in freshly diluted Oil Red O solution. Finally, cells were washed with running water and observed under the microscope.

***Glucose uptake assay***

Uptake of 2-deoxy-D-[$^3$H]glucose (Amersham Biosciences, Piscataway, NJ, USA) was measured as previously described. Briefly, 3T3-L1 adipocytes were first treated with or without 0.5μg/ml LPS overnight before the assay. Cells were then serum starved in KRH buffer supplemented with 0.5 % BSA and 2 mmol/L sodium pyruvate (pH 7.4) for 2 h before incubated either with or without 173 nmol/L insulin for 30 min at 37°C. Glucose uptake was initiated by adding 100 μmol/L [$^3$H] 2-deoxy-D-glucose. After 5 min, cells were washes with ice-cold PBS buffer for three times and then solubilized with KRH buffer containing 1% Triton X-100. Incorporated radioactivity was determined by scintillation counting.

***Statistical analysis***

Results are expressed as means ± SEM. Differences in the parameters between two groups were evaluated using Student's *t*-test with a 0.05 2-sided significance level. One way ANOVA was used to compare the differences between multiple groups. A value of $P < 0.05$ was considered significant.

# RESULTS

### Role of lysosomal activity in inflammation in adipocytes

To investigate the role of lysosomal activity in inflammation in adipocytes, we used BafA1 to inhibit lysosomal activity and evaluated the effect of BafA1 on the gene and protein expression of lysosomal proteins (CtB and CtL), the gene expression of inflammatory cytokines, and the activation of inflammatory signaling pathways in 3T3-L1 adipocytes. BafA1 is known to inhibit autophagy by impairing lysosomal hydrolase activity and preventing the fusion of autophagosome with lysosome [209]. Treatment with BafA1 for 24h led to an upregulation of CtB and CtL gene expression (Fig. 1A). At the protein level, BafA1 treatment completely blocked the maturation of pro-CtB as evidenced by a complete inhibition of the pro-CtB conversion to mature CtB (Fig. 1B). Interestingly, pro-CtB but not mature CtB was secreted into culture media; BafA1significantly increased pro-CtB secretion (Fig. 1C). BafA1 also stimulated CtL protein expression and secretion (Fig. 1B, C). BafA1 did not seem to significantly affect cellular NPC2 protein expression, but did slightly increased NPC2 secretion (Fig. 1B, C). To determine how inhibiting lysosomal activity affect inflammatory response, we

examined the gene expression of cytokines and inflammatory signaling pathway activation. As shown in Fig 1D, the phosphorylation of both NF-κB and ERK1/2 was increased after 24h treatment with BafA1. Moreover, the mRNA expression of TNFα, MCP1 (Fig 1E) and inducible nitric oxide synthase (iNOS) (Fig 1F) was significantly increased, while the mRNA expression of extracellular matrix (ECM) proteins matrix metallopeptidase 9 (MMP9), collagen 3 (Col3), and α-smooth muscle actin (αSMA) was decreased (Fig 1G). These results suggest that inhibiting lysosomal activity by BafA1 induces inflammatory response in adipocytes.

**Generation of stable NPC2 knockdown 3T3-L1 cell line**

To understand the role of NPC2 in adipocyte metabolism and function, we first examined the NPC2 gene expression during adipocyte differentiation. As shown in Fig. 2A, NPC2 expression was increased by more than one fold during differentiation, indicating that it plays more important roles in mature adipocytes than in adipocyte progenitors. Next, we generated NPC2 knockdown 3T3-L1 cells to study the role of NPC2 in regulating lysosomal activity and its relation to metabolism and inflammation in adipocytes. 3T3-L1 fibroblasts were transduced with lentivirus containing the shRNA targeting NPC2gene. Transduced cells were selected after a screening period of 7 days in antibiotics puromycin. Gene knockdown efficiency was examined at both mRNA and protein level. As shown in Fig 2B, lentiviral shRNA was able to knock down the gene expression of NPC2 by 60% in 3T3-L1 adipocytes. Moreover, the protein level of NPC2 was markedly reduced by more than 90% in NPC2 knockdown adipocytes compared to scrambled controls (Fig. 2B). To evaluate the possible effect of NPC2 knockdown on

adipocyte differentiation, Oil-red O staining as well as qPCR on gene expression levels of adipocyte markers were performed on scrambled and NPC2 KD 3T3-L1 adipocytes on Day 8 of differentiation. Oil Red-O staining results showed that both scrambled and NPC2 KD cells seemed to have similar differentiation capacity (Fig 2C). Additionally, the mRNA expression levels of adipocyte marker genes such as FABP4, C/EBPα and LPL were not different between scrambled and NPC2 KD adipocytes (Fig. 2D), whereas, the expression of PPARγ gene was decreased by 20% in NPC2 KD adipocytes (Fig. 2D).

**NPC2 knockdown impairs lysosomal activity in adipocytes**

NPC2 has been identified as an intracellular cholesterol transporter which acts in concert with NPC1 to remove cholesterol from the endosomal/lysosomal compartment. Previous studies on NPC1 function using NPC1-/- mice and cell models have demonstrated that there was a decrease in autophagy and lysosomal cathepsin activity in NPC1-/- fibroblasts, indicating that NPC1 plays an important role in autophagy-lysosomal system [210, 211]. Herein, we investigated how NPC2 deficiency affects lysosomal activity in adipocytes. First, we determined the lysomosal activity in scrambled and NPC2 KD 3T3-L1 adipocytes by measuring the protein levels of cathepsin B expression. We found that there was a significantly reduced expression of mature form of CtB in NPC2 KD adipocytes (Fig. 3A). MMP9, which was significantly accumulated by BafA1 treatment (Fig. 3B), can be used as an indicator of lysosomal function. As demonstrated in Fig 3C, MMP9 was increased in NPC2 KD adipocytes compared to scrambled cells, suggesting a defective lysosomal proteolysis in NPC2 KD adipocytes. Since lysosomal activity plays a key role in autophagic flux, we then looked at the protein

levels of LC3 and p62 in NPC2 KD adipocytes under the basal state of autophagy. Interestingly, we found that both LC3-I and LC3-II levels were significantly increased in NPC2 KD adipocytes when compared with scrambled cells (Fig. 3D); so were p62 levels (Fig. 3D). However, other autophagy-related proteins such as Beclin-1 and Atg7 were not altered in NPC2 KD cells (Fig. 3E). These results suggest that the activity of lysosomal degradation is impaired in NPC2 KD adipocytes.

**NPC2 knockdown reduces LPS effect on inflammatory response in adipocytes**

Autophagy-lysosome system has been known to regulate inflammatory response in immune cells. Our results from the experiments with lysosomal inhibitor above have linked lysosomal function to inflammation in adipocytes. We then investigated the effect of NPC2 knock down on inflammatory response in adipocytes. We showed that LPS treatment for 24h led to an increase in both the gene and protein expression of NPC2 in 3T3-L1 adipocytes (Fig. 4A and 4B), implying that NPC2 may play a role in LPS-induced inflammation.

We next investigated how NPC2 knock down impacts the effect of LPS on inflammation. As shown in Fig. 4C, NPC2 KD adipocytes reduced NF-κB and ERK1/2 phosphorylation in response to LPS stimulation compared to scrambled adipocytes. LPS treatment for 24h significantly stimulated NF-κB and ERK1/2 phosphorylation in scrambled adipocytes, but this effect was diminished in NPC2 KD adipocytes (Fig. 4C). Consistently, the LPS-induced expression of inflammatory genes including TNFα, IL18, MCP1 and MMP9 was also significantly attenuated in NPC2 KD adipocytes (Fig. 4D). In addition, we found that LPS did not seem to induce the expression of ECM genes such as

fibronectin (FN), Col1, Col3, Col4, and αSMA in scrambled adipocytes. Interestingly, the mRNA expression of these ECM genes was downregulated in NPC2 KD adipocytes under the basal condition when compared with scrambled controls (Fig. 4E). Moreover, LPS significantly upregulated the expression of ECM (FN, Col1, Col3 and Col4) in NPC2 KD adipocytes but not scrambled cells (Fig 4E). Additionally, LPS was able to stimulate TLR2 and TLR4 expression to a similar extent in both scrambled and NPC2 KD adipocytes (Fig. 4F).

**NPC2 knockdown diminishes LPS-stimulated glucose uptake, but does not affect insulin sensitivity in adipocytes**

Our results have shown that NPC2 knockdown adipocytes have reduced basal lysosomal activity and inflammatory response to LPS stimulation. We then determined the effect of NPC2 knockdown on glucose metabolism and insulin sensitivity in adipocytes. As illustrated in Fig 5A, NPC2 KD adipocytes had slightly increased basal insulin-stimulated glucose uptake. Interestingly, 24h LPS treatment caused increased glucose uptake in scrambled adipocytes, but this stimulatory effect of LPS was completed blunted in NPC2 KD adipocytes (Fig 5B). The treatment of 1 μg/ml insulin for 30min induced the phosphorylation of Akt at Ser 473 to a similar extent in both scrambled and NPC2 KD adipocytes (Fig 5C). Moreover, while the mRNA expression of GLUT4 and adiponectin was not different between scrambled and NPC2 knockdown adipocytes under the basal condition, the mRNA expression of leptin was significantly reduced by more than 60% in NPC2 KD adipocytes compared to scrambled cells(Fig 5D).

# DISCUSSION

NPC2 is known as a protein in the late endosome/lysosome, and functions as a cholesterol transporter in concert with NPC1. While most of the previous studies on NPC2 focused on its role in Niemann-Pick type C disease, the physiological function of this protein remains elusive. Our previous studies have suggested a connection of adipose NPC2 to obesity [207]. In a previous study from others, the role of NPC2 in adipocyte differentiation and function has been investigated using differentiated adipocytes from human skin fibroblasts as a model of adipocytes [212]. In this study, we focused on different aspects and investigated the role of NPC2 in regulating lysosomal activity in adipocytes, and the impact of NPC2 KD on adipocyte inflammation and function.

First, we determined the effect of lysosomal activity on inflammation in adipocytes. Treatment with the lysosomal inhibitor BafA1 blocks the conversion of pro-CtB to mature CtB, leading to increased pro-CtB accumulation and secretion in adipocytes. BafA1 also causes an increase in CtL, another lysosomal protease. Moreover, BafA1 treatment increases pro-inflammatory signaling pathway activation as evidenced by the increased NF-κB and ERK1/2 phosphorylation as well as the upregulation of TNFα, MCP1, and iNOS gene expression. These results suggest that inhibiting lysosomal function results in adipocyte stress and inflammation. In NPC2 KD adipocytes, we found that the mature form of CtB protein was reduced. The protein level of MMP9, which was increased by BafA1, was also upregulated in NPC2 KD adipocytes. Consistently, NPC2 KD led to increased p62 and LC3 I/II protein levels under the basal condition, indicating that NPC2 knockdown impairs lysosomal proteolytic activity and function. Our results

are in line with the previous studies investigating the changes of autophagy in NPC disease. Both NPC1 deficiency and U18666A, which similarly inhibits the efflux of cholesterol from lysosome, led to enlarged autophagosomes, impaired autophagy flux and accumulation of ubiquitinated proteins in the endosomal/lysosomal compartment [213, 214]. Therefore, our results support the fact that NPC2 plays an important role in maintaining normal autophagy-lysosomal activity.

In a recent study, NPC2 has been shown to contain MD-2-related lipid-recognition (ML) domain; secreted drosophila NPC2a, a homologous of vertebrate NPC2 in function, can bind to bacterial lipopolysaccharide (LPS) and modulate LPS effect on inflammatory signaling pathways [215]. However, similar effects of NPC2 have not been reported in mammalian cells. Therefore, we were interested in the role of NPC2 in mediating LPS effect on inflammation in adipocytes. We demonstrated that LPS is able to induce both the gene and protein expression of NPC2 in adipocytes, and NPC2 deficiency impairs the lysosomal activity. We next studied how NPC2 deficiency affects LPS-induced adipocyte inflammation and function. Our results from the study of adipocyte cultures showed that 24-hour treatment of LPS significantly induces the phosphorylation of NF-κB and ERK1/2 in scrambled adipocytes, but fails to do so in NPC2 KD adipocytes. This suggests that knocking down NPC2 reduces LPS-induced inflammatory response. To provide additional evidence supporting this conclusion, we examined the effect of LPS on the gene expression of cytokines and ECMs in NPC2 KD adipocytes. We found that NPC2 KD does not affect basal levels of the mRNA expression of inflammatory cytokines and chemokines such as TNFα, IL-6, IL-18, and MCP1, but significantly reduces the mRNA expression of several ECM proteins

including MMP9, FN, Col1, Col3, Col4 and αSMA. More interestingly, NPC2 KD adipocytes have reduced response to LPS stimulation in the mRNA expression of cytokines. All the data together supports the fact that NPC2 deficiency impairs LPS effect on inflammation in adipocytes.

It is of interest to investigate how NPC2 KD affects adipocyte metabolism and function. We then examined glucose uptake, insulin sensitivity and adipokine expression in NPC2 KD adipocytes. The basal level of glucose uptake was slightly increased in NPC2 KD adipocytes compared to scrambled adipocytes. Insulin is able to stimulate glucose uptake in both scrambled and NPC2 KD adipocytes at the similar level. Consistently, insulin-stimulated Akt phosphorylation was not different between scrambled and NPC2 KD adipocytes, suggesting that NPC2 KD does not interfere with insulin sensitivity. However, NPC2 KD diminishes LPS-induced glucose uptake in adipocytes, which is consistent with the earlier result showing that NPC2 KD blunted LPS induced inflammatory response. The similar levels of expression of TLR2 and TLR4 genes between scrambled and NPC2 KD adipocytes in the basal and LPS-stimulated condition suggests that the effect of NPC2 KD on reducing LPS-stimulated inflammation may be through a mechanism independent of TLR transcription.

There exist several lines of evidence that support a potential new model for the role of NPC2 in mediating LPS induction in inflammation. First, a previous study described the similarity between the cholesterol binding domain in NPC2 and the lipid-recognition domain in MD-2, which is an extracellular binding partner of TLR4 and essential for the LPS-induced innate immune response [216, 217]. A later study on

secreted drosophila NPC2a, a homolog of vertebrate NPC2 in function, has shown that NPC2a functions similarly to MD-2; it binds to the lipid A moiety of bacterial lipopolysaccharide (LPS) and modulates LPS effect on inflammatory signaling pathways [215]. It is reasonable to speculate that NPC2 has a similar effect in mammalian cells. Different from this secretory drosophila NPC2 model, we found that the majority of NPC2 protein seems to be located intracellularly in 3T3-L1 adipocytes. We only detected a very limited level of NPC2 in culture media. Therefore, it is likely that the endosome/lysosomal NPC2, but not the secretory NPC2 is the major regulator of inflammatory response in adipocytes upon LPS stimulation. Indeed, there is an established pathway by which LPS triggers immune response in the endosome. Different from the TIRAP and MyD88 pathway, which mediates the signaling from TLR4 on the plasma membrane, internalized LPS and TLR4 in the endosome trigger inflammatory response via a different signaling pathway that involves TRAM and TRIF [218, 219]. These adaptors in turn activate the transcription factor interferon regulatory factor-3 (IRF3), which regulates type I interferon (IFN) expression [220] and involves the late phase of NF-κB activation. The activated NF-κB is then translocated into the nucleus to initiate the transcription of pro-inflammatory cytokines. Based on our data and the above information from the literature, we propose that NPC2 may be required as a potential co-activator for the activation of TLRs on the endosomal membrane and its signaling transduction to NF-κB pathway. Therefore, NPC2 deficiency blocks the activation of NF-κB by LPS stimulation. To confirm this hypothesis, further studies are needed to examine the interaction of LPS, NPC2 and TLR4 in the endosome as well as the activity of TRAM-TRIF pathway in NPC2 deficient adipocytes.

In summary, we have demonstrated that NPC2 knockdown impairs lysosomal activity in adipocytes. We also found that NPC2 knockdown blunts LPS effect on inflammation and glucose uptake in adipocytes. We propose that NPC2 is an important regulator of the LPS-TLR4 signaling pathway activation that takes place in endosomes. Our studies indicate that NPC2 could serve as a potential therapeutic target for treating adipose tissue inflammation.

## FIGURES

## Figure 1. BafA1 regulates lysosomal protein expression and induces inflammation in 3T3-L1 adipocytes.

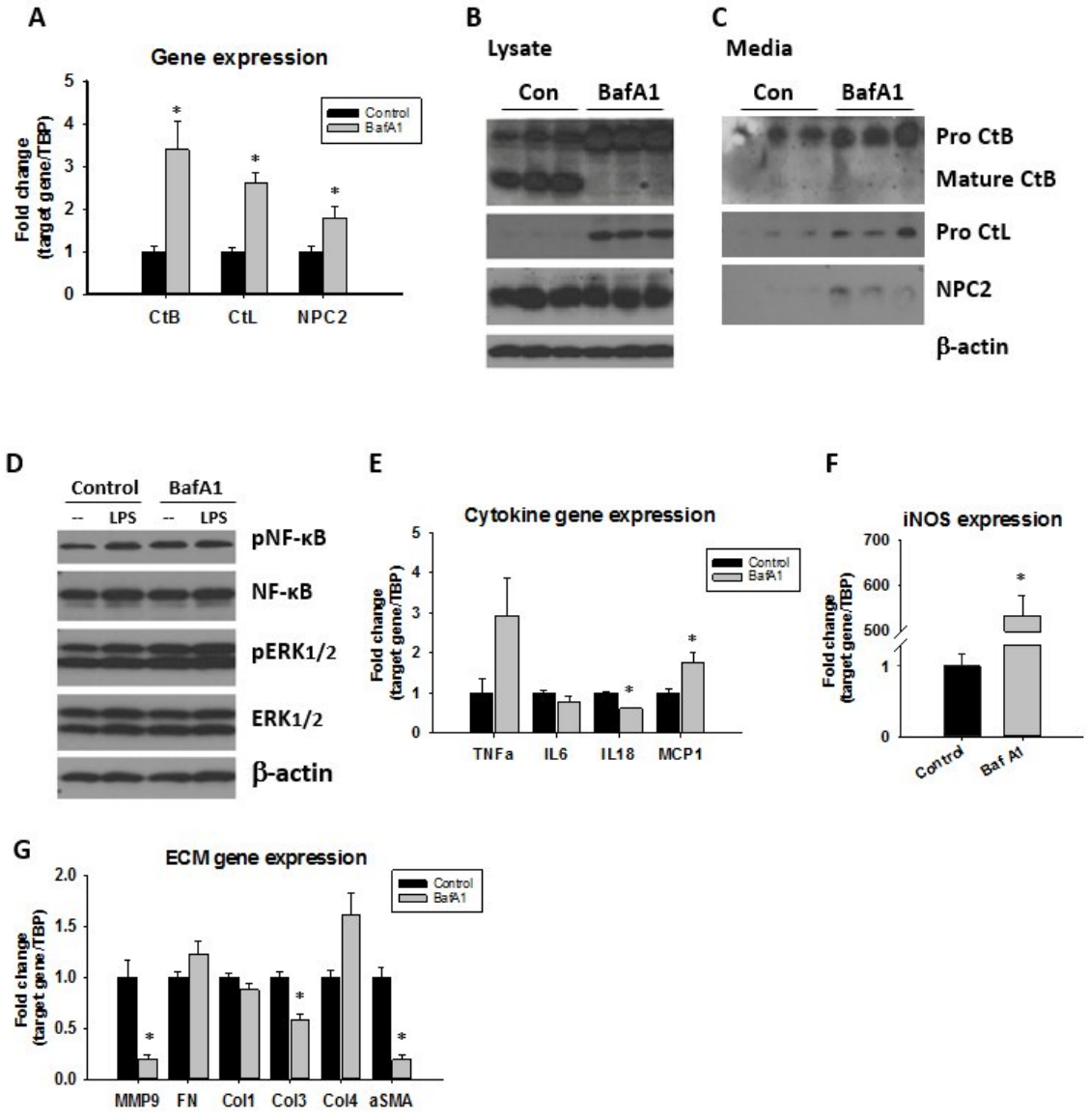


**Figure 2. Generation of stable NPC2 knockdown 3T3-L1 cell line.**

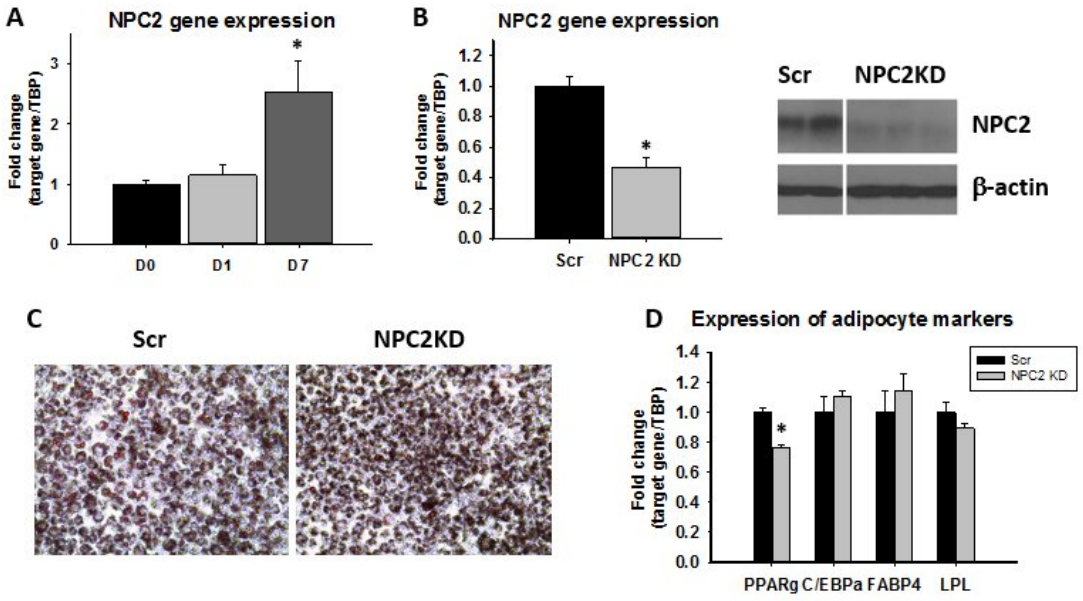


**Figure 3. NPC2 knockdown impairs lysosomal activity in adipocytes.**

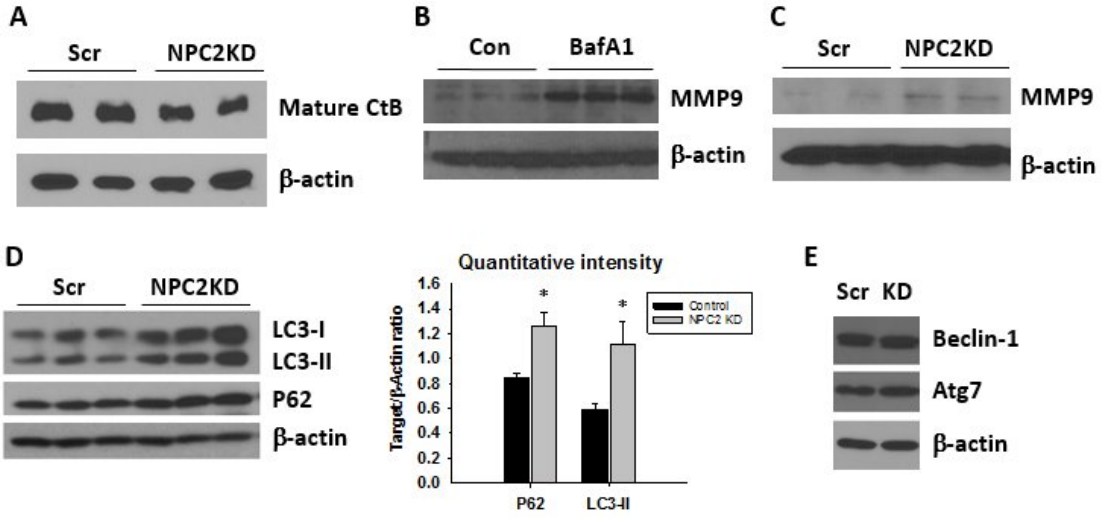


**Figure 4. NPC2 knockdown reduces LPS effect on inflammatory response in adipocytes.**

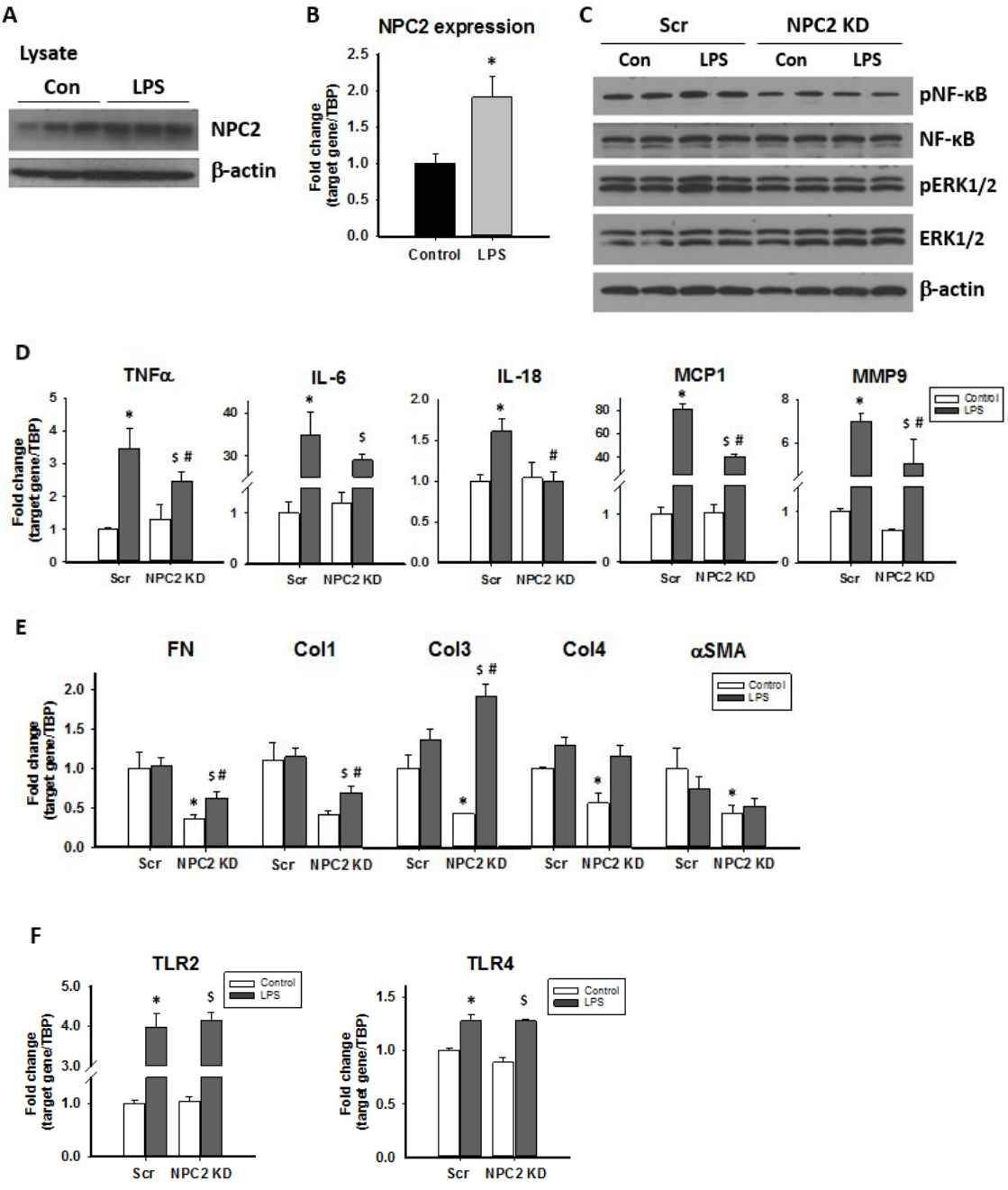


**Figure 5. Effect of NPC2 knockdown diminishes LPS-stimulated glucose uptake and insulin sensitivity in adipocytes.**

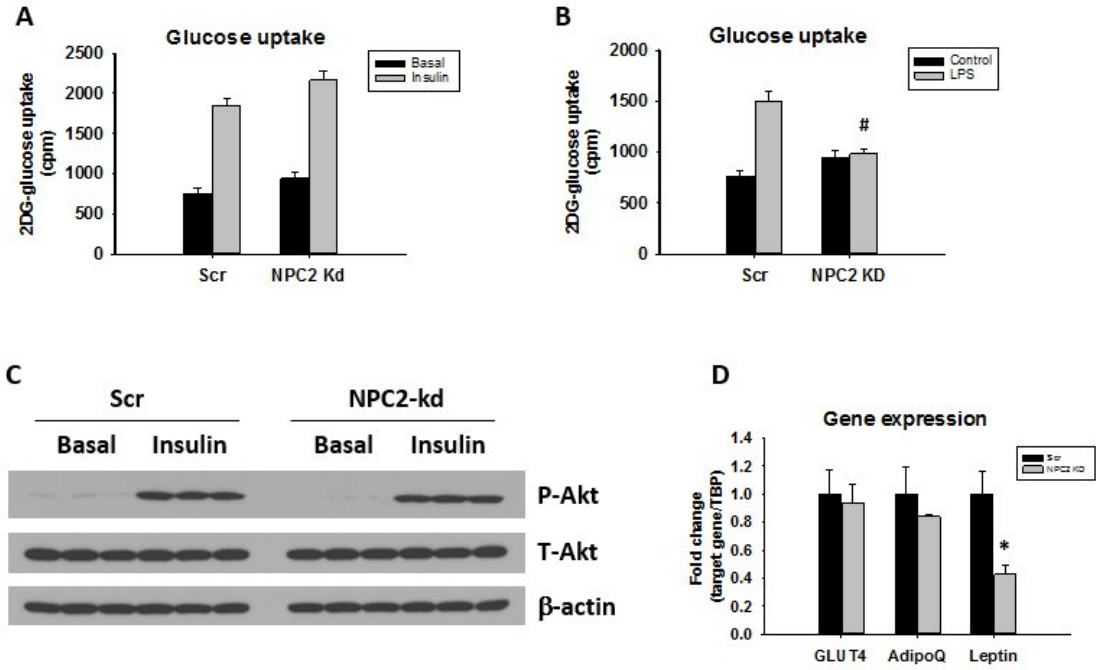
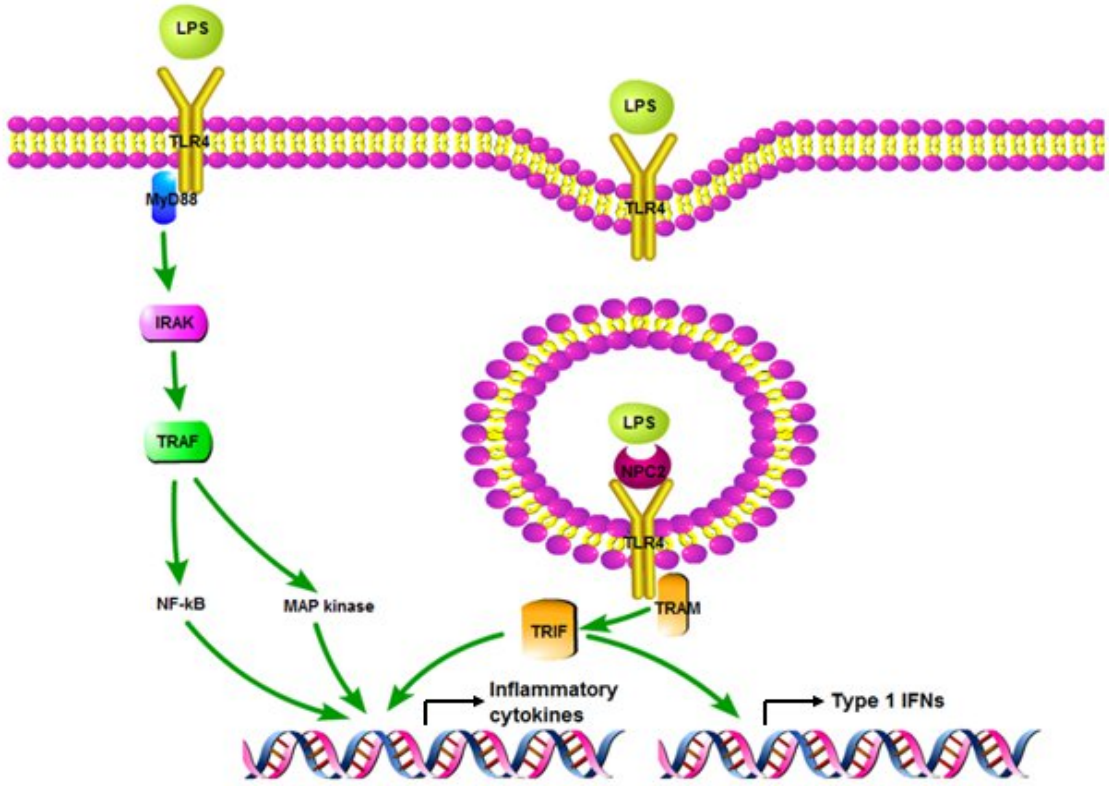


**Figure 6. A model of NPC2 role in LPS signaling in adipocytes.**



## FIGURE LEGEND

**Figure 1. BafA1 regulates lysosomal protein expression and induces inflammation in 3T3-L1 adipocytes.**

Fully differentiated 3T3-L1 adipocytes were treated with 100nM BafA1 for 24 hours. The mRNA expression of Cathepsin B (CtB), CtL, and NPC2 (A) were determined by real-time PCR. Protein levels of these three genes in cell lysate (B) and culture media (C) were measured by Western Blotting. 3T3-L1 adipocytes were either treated with BafA1 or 0.5μg/ml LPS alone, or together for 24 hours. NF-κB and ERK1/2 phosphorylation (D) were determined by Western Blotting. The mRNA expression of cytokines (E), iNOS (F) and ECM genes (G) was measured in 3T3-L1 adipocytes after 24-hour BafA1 treatment. The 3-4 wells of cells were included for each treatment group. The experiment was repeated 2-3 times with different sets of cells. The data are represented as mean ± SEM. * $p<0.05$, compared with control.

**Figure 2. Generation of stable NPC2 knockdown 3T3-L1 cell line.**

Post-confluent 3T3-L1 cells were induced to differentiate into adipocytes. A: the mRNA expression of NPC2 in 3T3-L1 adipocytes on D0, D1 and D7 of differentiation. NPC2 gene was knockdown in 3T3-L1 fibroblasts by shRNA. B: NPC2 gene (left) and protein (right) expression in scrambled and NPC2 knockdown adipocytes were measured by real-time PCR and Western Blotting, respectively. Oil-red O staining (C) and the expression

of adipocyte marker genes (D) were detected in scrambled and NPC2 knockdown 3T3-L1 adipocytes.

**Figure 3. NPC2 knockdown impairs lysosomal activity in adipocytes.**

The protein expression of mature CtB (A) in differentiated scrambled and NPC2 KD 3T3-L1 adipocytes was measured by Western Blotting. The MMP9 protein in adipocytes treated with 100nM BafA1 for 24 hours (B) or in scrambled and NPC2 knockdown adipocytes (C) were determined by Western Blotting. Autophagic markers LC-3, P62 (C) and Beclin-1, Atg7 (D) in scrambled and NPC2 knockdown adipocytes were measured by Western Blotting. The experiment was repeated 2-3 times with different sets of cells. The data are represented as mean ± SEM. * $p<0.05$, compared with control.

**Figure 4. NPC2 knockdown reduces LPS effect on inflammatory response in adipocytes.**

Protein (A) and mRNA expression (B) of NPC2 in differentiated 3T3-L1 adipocytes treated with 0.5μg/ml LPS for 24-hour. Scrambled and NPC2 KD adipocytes were treated with 0.5μg/ml LPS for 24-hour. NF-κB and ERK1/2 phosphorylation (C) was determined by Western Blotting; the mRNA expression of pro-inflammatory cytokines (D), ECM genes (E) and TLRs (F) was measured by real-time PCR. The experiment was repeated 2-3 times with different sets of cells. The data are represented as mean ± SEM. *

$p<0.05$, compared with control of scrambled cells, $^{\$}$ $p<0.05$, compared with control of NPC2 KD cells, $^{\#}$ $p<0.05$, compared with scrambled cells treated with LPS.

**Figure 5. Effect of NPC2 knockdown diminishes LPS-stimulated glucose uptake and insulin sensitivity in adipocytes.**

Insulin-stimulated uptake of [$^{3}$H]-2 deoxy-D-glucose in scrambled and NPC2 KD adipocytes (A) and glucose uptake in adipocytes with 0.5μg/ml LPS treatment for 24-hour (B). Insulin-induced Akt phosphorylation was measured in scrambled and NPC2 KD adipocytes (C) by Western Blotting. The mRNA expression of GLUT4, adiponectin and leptin in scrambled and NPC2 KD adipocytes was determined by real-time PCR (D). The experiment was repeated 2-3 times with different sets of cells. The data are represented as mean ± SEM. * $p<0.05$, compared with control, $^{\#}$ $p<0.05$, compared with scrambled cells treated with LPS.

**Figure 6. A model of NPC2 role in LPS signaling in adipocytes.**

# CHAPTER 4

# EICOSAPENTAENOIC ACID PROMOTES THERMOGENIC AND FATTY ACID STORAGE CAPACITY IN MOUSE SUBCUTANEOUS ADIPOCYTES

This chapter is modified from the published paper:

Zhao M and Chen X. Eicosapentaenoic acid promotes thermogenic and fatty acid storage capacity in mouse subcutaneous adipocytes. *Biochemical and Biophysical Research Communications* 450(4):1446-51, 2014.

## SUMMARY

In this study, we determined if eicosapentaenoic acid (EPA) promotes beneficial metabolic activities of subcutaneous adipocytes. Stromal-vascular (SV) cells were isolated from inguinal adipose tissue of C57BL/6 mice and induced to differentiate into adipocytes. EPA effect on thermogenic and mitochondrial gene expression and oxidative metabolism were assessed in inguinal adipocytes. When added to SV cell cultures during 8 day differentiation, EPA significantly increased the expression of thermogenic genes UCP1-3, CIDEA and VEGFα. Moreover, EPA increased mitochondrial DNA content and the expression of genes involved in mitochondrial biogenesis including PGC1α, Nrf1 and COXiv. However, this effect was not evident when EPA was added to mature inguinal adipocytes for 24hr, suggesting that EPA exerts its browning effect via recruiting beige adipocytes. Consistently, long-term EPA treatment also upregulated AMPKα phosphorylation and CPT1 expression and increased glucose uptake and GLUT4 mRNA expression, suggesting improved mitochondrial oxidation. Additionally, EPA-treated adipocytes had enlarged lipid droplets and increased expression of triglyceride synthesis genes GPAT1 and GPAT3, while significantly decreased glycerol release and down-regulation of HSL and ATGL gene expression. We conclude that EPA enhances energy dissipation capacity by recruiting beige adipocytes to stimulate oxidative metabolism and reduces fatty acid release by facilitating fatty acid storage in subcutaneous adipocytes.

# INTRODUCTION

Obesity is a serious health problem in the United States. Adipose tissue dysfunction, which occurs in obesity, is the major contributor to obesity-associated metabolic complications such as hypertension, cardiovascular disease, inflammation, insulin resistance, and type 2 diabetes. Two types of adipose tissues exist and exert opposite metabolic functions. Brown adipose tissue (BAT) is the major depot that plays a role in thermogenesis and energy expenditure, while white adipose tissue (WAT) mainly stores lipids and secretes metabolic regulators of adipokines and cytokines.

In recent years, researchers found that brown-like adipocytes (beige adipocytes) can be recruited in white adipose tissue in response to cold and β-adrenergic stimulation [19, 20, 221]. This browning process is regulated by multiple transcription factors, co-activators, and other molecular regulators, including PR domain containing 16 (PRDM16), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), peroxisome proliferator-activated receptor (PPARs), cell death-inducing DFFA-like effector a (CIDEA) and CCAAT-enhancer-binding protein-beta (C/EBPβ) [41-43, 222]. More importantly, the browning process also potentially protects against obesity and insulin resistance [37, 223]. It has long been known that there exist depot differences in metabolic functions of WAT. Recent studies suggest that subcutaneous white adipose tissue (SC-WAT) has higher lipid storage capacity as well as thermogenic potential when compared with visceral WAT [43, 51]. These characteristics of SC-WAT may contribute to its metabolic benefits [140].

Mitochondrion is a key organelle responsible for substrate oxidation and energy dissipation. Recent studies have emphasized the importance of mitochondrial function, especially in SC adipocytes in the development of insulin resistance [224]. In obesity, mitochondrial dysfunction in adipocytes leads to the production of toxic lipid species, inflammation and insulin resistance [225, 226]. Therefore, enhancing mitochondrial function and fatty acid oxidation, as well as increasing lipid storage capacity of adipocytes could lead to decreased release of free fatty acids (FFAs) from adipocytes, thereby preventing the circulating FFA elevation and subsequently metabolic deterioration.

Polyunsaturated fatty acids (PUFAs) of the n-3 series have been known for their beneficial effects in regulating adipose tissue function. Three major members of n-3 FAs include α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Previous studies on the metabolic effects of n-3 FAs showed that n-3 FAs are able to lower serum triglyceride (TG), reduce chronic inflammation, and increase insulin sensitivity in animals and human subjects [227-230]. The activation of PPARα, PPARγ and AMP-activated protein kinase (AMPK) could be the pathways that mediate the effect of n-3 FAs [231-234]. However, most of previous studies were performed in animal models or 3T3-L1 cell line without distinguishing the role of n-3 FAs in different fat depots.

In this study, we specifically investigated the metabolic effect of EPA on SC adipocytes and determined whether EPA promotes beneficial metabolic activities in primary differentiated SC adipocytes. We found that EPA treatment during the process of

inguinal adipocyte differentiation significantly increased the expression of thermogenic genes and mitochondrial biogenesis/ function as well as TG storage.

## METHODS AND MATERIALS

### *Animals*

Animal handling followed the U.S. National Institutes of Health guidelines, and experimental procedures were approved by the University of Minnesota Animal Care and Use Committee. C57BL/6 mice purchased from Jackson Laboratory were housed in specific pathogen-free facility at the University of Minnesota. Mice were allocated into groups (3 or 4 mice/cage) and fed a regular chow diet (RCD) with free access to water.

### *Cell culture*

Stromal-vascular (SV) cells were isolated from inguinal WAT of C57BL/6 mice as described previously [167]. After mincing, fat pads were digested with collagenase (2 mg/ml solution) in Krebs-Ringer bicarbonate HEPES (KRBH) buffer. After 1.5h digestion, SV cells were separated by centrifugation at 1500 rpm for 10 min and then cultured in growth medium (DMEM plus 10% FBS) until confluence. Cells were then treated with a differentiation cocktail containing 10% fetal bovine serum, 115 μg/ml methylisobutylxanthine, 390ng/ml dexamethasone, and 1 μg/ml insulin for 3 days. Three days later, the differentiation cocktail was replaced with DMEM containing 10% fetal bovine serum, 100IU/mL penicillin/streptomycin, 1μg/ml insulin, and cultures were

continued for another 5 days. During 8 days of differentiation, 200μM EPA (EMD Chemicals, NJ, USA) was added to the cultures as fatty acid/fatty acid-free bovine serum albumin (BSA) complexes. The molar ratio of fatty acid to BSA was 4:1. FA-free BSA was used as a control.

***Western blotting***

Proteins were extracted from cell samples in RIPA buffer (Sigma, St. Louis, MO, USA) containing protease inhibitors. Equal amounts of proteins were separated on 10% SDS-PAGE gel and transferred to polyvinylidene fluoride membrane. The membranes were probed with phospho-AMPKα (Thr172), AMPKα or β-Actin (Cell Signaling, MA, USA) antibodies according to the recommendations of the manufacturers. ECL Western Blotting Detection Systems (GE Healthcare BioSciences, Piscataway, NJ, USA) were used to detect antibody reactivity. The density of bands was quantified by Image J software, and the results were normalized to β-Actin of each corresponding samples.

***Quantitative real-time RT-PCR***

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Real-time PCR was performed using SYBR Green qPCR Master Mix (SABioscience, Frederick, MD, USA) with an ABI Step One Plus real-time PCR System (Applied Biosystems, Foster City, CA, USA). Primer sequences are provided in Table 3. Results are normalized to β-actin using the $\Delta\Delta C_t$ method and presented as levels of expression relative to that of controls.

***Mitochondrial DNA content***

Total DNA was extracted as previously described [24]. The samples were digested in lysis buffer (0.01 M Tris–HCl, 0.25 mM EDTA, 0.5% SDS, pH 8.3) containing 100 μg/ml proteinase K and extracted with chloroform and 8 M potassium acetate. The supernatant was collected and precipitated with isopropyl alcohol and ethanol. mtDNA content was determined by detecting DNA levels of cytochrome C oxidase 2 (COX2) using real-time PCR. The value was normalized to the levels of nuclear genome DNA RIP140.

***Glucose uptake assay***

Uptake of [$^3$H] deoxy-D-glucose (Amersham Biosciences, Piscataway, NJ, USA) was measured as previously described [9, 235]. Briefly, cells were serum starved in KRH buffer supplemented with 0.5 % BSA and 2 mmol/L sodium pyruvate (pH 7.4) for 3 h at 37°C. Glucose uptake was initiated by adding 100 μmol/L [$^3$H]-deoxy-D-glucose. After 5 min, and the cells were solubilized with KRH buffer containing 1% Triton X-100 after three washes with ice-cold PBS buffer. Incorporated radioactivity was determined by scintillation counting.

***Measurements of glucose, lactate and glycerol levels***

Glucose and lactate levels in cell culture medium were detected with Autokit Glucose (Wako, VA, USA) and lactate Assay Kit (Sigma-Aldrich, MO, USA), respectively. Media glycerol levels were detected with Free Glycerol Reagent (Sigma Aldrich, Saint Louis, MO) following the instruction provided by manufactures.

***Triglyceride content measurement***

On day 8 of differentiation, adipocytes were fixed in cold Baker's Formalin for 30 minutes at 4°C, followed by staining in Oil Red O for 10 minutes. After rinsing the wells with water, isopropanol was added to each well to elute the dye for 15 minute. Then the solvent was added into a 96 well plate and the OD was read at 540nm.

***Statistical analysis***

Results are expressed as means ± SEM. Differences in the parameters between groups were evaluated using Student's *t*-test with a 0.05 2-sided significance level. A value of $P < 0.05$ was considered significant.

# RESULTS

**EPA regulates thermogenic gene expression in inguinal adipocytes**

As shown in Fig. 1A, 8 day of EPA treatment had no evident effect on inguinal adipocyte differentiation. This morphological result was confirmed by the gene expression of PPARγ (Fig. 1B). Interestingly, 8 day treatment of EPA during the adipocyte differentiation process significantly increased gene expression of UCP1-3(Fig. 1C). Similar effect was observed in differentiated brown adipocytes (Fig. 1D). In addition, EPA significantly increased CIDEA but not PRDM16 and C/EBPβ expression in inguinal adipocytes (Fig. 1E). Moreover, our results showed that there was a 2-fold increase in vascular endothelial growth factor α (VEGFα) in EPA-treated inguinal adipocytes (Fig. 1E). VEGFα is a key pro-angiogenic factor and its upregulation is an

essential process for thermogenic activation of BAT [236]. Taken together, our data suggest a browning effect of EPA in inguinal adipocytes.

**Effect of EPA on mitochondria biogenesis and oxidation in brown and inguinal adipocytes**

To evaluate the effect of EPA on mitochondrial biogenesis and oxidation capacity, mitochondrial DNA (mtDNA) content and mitochondrial oxidation gene expression were determined in brown and inguinal adipocytes. EPA treatment during the adipocyte differentiation process significantly increased mtDNA content in inguinal and brown adipocytes (Fig. 2A). Consistently, the expression of genes involved in mitochondrial biogenesis and oxidation such as PGC1α, Nuclear respiratory factor 1 (Nrf1) and cytochrome c oxidase iv (COXiv) were significantly up-regulated in inguinal adipocytes when EPA was added during 8 day of differentiation (Fig. 2B).

Mitochondrial function is an important factor that determines the fate of glucose metabolism, i.e. through mitochondrial oxidation or glycolysis. Therefore, lactate levels can be indicative of glucose oxidation level and mitochondrial function. As illustrated in Fig. 2C, EPA-treated inguinal adipocytes in the presence of insulin for 24hr had increased glucose uptake by approximately 30% compared to control cells. We also showed that the gene expression of GLUT4, but not GLUT1 was markedly upregulated by EPA treatment (Fig. 2D), suggesting that EPA increases glucose uptake by increasing GLUT4 expression. The glucose concentration in 24 hour cultured conditional medium was significantly lower in EPA-treated ingunal and brown adipocytes compared to control cells (Fig. 2E), which consistently reflects increased uptake of glucose by the

cells. However, lactate levels in the media were similar between control and EPA-treated inguinal and brown adipocytes (Fig. 2F). This implies that the additional glucose that has been taken-up into the cells is metabolized possibly through the oxidation pathway, supporting increased mitochondrial oxidation by EPA in adipocytes.

To provide additional evidence supporting the enhanced mitochondrial function by EPA, we examined the EPA effect on FA oxidation. As shown in Fig. 3A and 3B, EPA treatment led to the upregulation of lipoprotein lipase (LPL), Cluster of Differentiation 36 (CD36), Carnitine palmitoyltransferase I (CPT1) gene expression, while down-regulation of acetyl-CoA carboxylase-alpha (ACCα) expression. Moreover, EPA treatment increased the phosphorylation of AMPKα at Thr172 residue (Fig. 3C). Together with an increase in PGC1α expression, all of these changes indicate an enhanced FA oxidation in inguinal adipocytes with 8 day treatment of EPA.

**EPA treatment during the differentiation process increases lipid storage capacity in inguinal adipocytes**

We next determined the EPA effect on lipid storage capacity in inguinal adipocytes. As shown in Fig. 4A, EPA-treated inguinal adipocytes had significantly larger lipid droplets than control cells, which is consistent with the increased triglyceride content in EPA-treated adipocytes (Fig. 4B). Moreover, the expression of Glycerol-3-Phosphate Acyltransferase 1 (GPAT1) and GPAT3 genes was upregulated by ~80% (Fig. 4C), suggesting an increase in TG synthesis in EPA-treated adipocytes. In addition to TG synthesis, lipolysis determines the TG content and lipid droplet size. Interestingly, EPA treatment reduced glycerol release into medium by 70% (Fig. 4D). This inhibitory effect

of EPA on lipolysis was only observed under the basal condition; EPA had no effect on norepinephrine-stimulated lipolysis (data not shown). In agreement with decreased glycerol release, EPA significantly inhibited the expression of HSL (hormone sensitive lipase) and ATGL (adipose triglyceride lipase) genes (Fig. 4E). However, EPA had no effect on perilipin1 gene expression (Fig. 4E). These data suggest that EPA promotes lipid storage capacity by increasing TG synthesis and decreasing lipolysis in inguinal adipocytes.

**Palmitate and DHA had no effects on thermogenic gene expression in brown and inguinal adipocytes**

Our results have clearly suggested that EPA increases mitochondrial function, FA oxidation, and lipid storage capacity in inguinal adipocytes. In terms of the specificity of EPA beneficial effect, we performed similar experiments using saturated fatty acid such as palmitate. Since chronic (8 day) treatment of palmitate is toxic to cells, we only looked at the effect of palmitate treatment for 24h in brown adipocyets. Unlike EPA, 24h treatment of palmitate had no significant effect on the expression of thermogenic genes including UCP1, PRDM16, C/EBPβ, and PGC-1α, while 24h EPA treatment significantly increased the expression of UCP1, PRDM16, and C/EBPβ in brown adipocytes (Fig. S1A). Furthermore, we conducted a similar experiment to examine the effect of DHA. Different from EPA, treatment of DHA during 8 day of adipocyte differentiation had no effect on thermogenic gene expression (Fig. S1B). DHA significantly inhibited adipocyte differentiation (Fig. S1C); this result is in line with a previous study in 3T3-L1 adipocytes [237].

# DISCUSSION

Recent studies have demonstrated that brown-like adipocytes can be recruited in white adipose tissue, particularly in subcutaneous fat depot, which holds a potential to prevent obesity [223]. Herein, we sought to investigate the effect of EPA on metabolic functions of subcutaneous adipocytes focusing on thermogenesis and lipid storage properties. We found that EPA treatment during inguinal adipocyte differentiation increases thermogenesis, mitochondrial oxidation, and lipid storage capacity in inguinal adipocytes.

N-3 PUFAs have been reported to increase the expression levels of UCPs in multiple tissues, such as brown adipose tissue, liver, and muscle [238-241]. However, few studies were focused on the browning effect of EPA on SC-WAT depot. In this study, we found EPA treatment increases the gene expression of UCP1-3 in inguinal adipocyte when added during the differentiation process. This, together with the up-regulation of CIDEA, VEGFα and PGC-1α expression, suggests the enhancement of thermogenesis and energy expenditure in SC adipocytes by EPA.

Mitochondrial function in white adipocytes determines lipid homeostasis and insulin sensitivity [224]. It directly regulates glucose and fatty acid oxidation, and also impacts other metabolic pathways, such as lipogenesis, lipolysis and FA re-esterification. Hence, we determined the EPA effect on mitochondrial biogenesis and oxidation capacity in adipocytes. Our results showed that EPA increased mtDNA content and the expression of mitochondrial oxidation genes, such as PGC1α, Nrf1 and COXiv. Consistently, changes in ACCα and CPT1 expression, along with increased AMPK phosphorylation, suggest

that FA oxidation is enhanced in EPA-treated adipocytes. Our results are in agreement with previous reports that mice fed a diet rich in DHA and EPA had enhanced FA oxidation and mitochondrial function in adipose tissue [242]. Additionally, our result of glucose metabolism implies that EPA enhances glucose oxidation, which may result from the improved mitochondrial oxidation.

The impairment of lipid storage function of white adipose tissue has been directly associated with the development of insulin resistance. The release of excessive fatty acids into the circulation due to decreased lipid storage capacity could be an important contributor to the ectopic fat accumulation and insulin resistance [140]. On the other hand, an increase of fat accumulation capacity in subcutaneous adipose tissue has been associated with the improvement of insulin sensitivity [243, 244]. In this study, we found that EPA promotes lipid storage capacity of inguinal adipocytes by increasing triglyceride synthesis and inhibiting lipolysis, which is consistent with previous reports showing that diets rich in fish oil reduces lipolysis and improves TG storage in adipose tissue [230, 231]. Taken together, our results suggest that stimulating thermogenic and oxidative metabolism of subcutaneous adipocytes, as well as increasing their lipid storage capacity, are some of the beneficial metabolic effects of EPA.

It seems contradictory that EPA inhibits lipolysis and increases FA oxidation simultaneously in the same adipocyte. However, we hypothesize that EPA may affect lipid storage and thermogenesis in two different types of adipocyte, i.e. white adipocyte and brown-like adipocyte (beige adipocyte), respectively in subcutaneous SV cell cultures. On the one hand, EPA increases thermogenic capacity by increasing the

recruitment of beige adipocytes from adipocyte progenitor cells in white adipose tissue, and our data supports this notion. For instance, the effect of EPA on thermogenic gene expression was perceived only when EPA was added during SV cell differentiation, but not in fully differentiated inguinal adipocytes treated with EPA for 24h (data not shown). On the other hand, EPA promotes lipid storage in white adipocytes by inhibiting lipolysis and increasing TG synthesis. Further investigations are needed to test this hypothesis.

In summary, our study provides evidence of a beneficial metabolic effect of EPA in inguinal adipocytes. Our results show that EPA induces mitochondria biogenesis, increases glucose uptake, and promotes oxidation of FAs and glucose. On the other hand, EPA enhances fatty acid uptake, lipid storage, but inhibits lipolysis, leading to decreased FA release. All of these changes together could be the mechanism by which EPA reduces inflammation and inhibits ectopic fat accumulation, thereby preventing insulin resistance.

**FIGURES**

## Figure 1. EPA effect on thermogenic gene expression in inguinal and brown adipocytes.

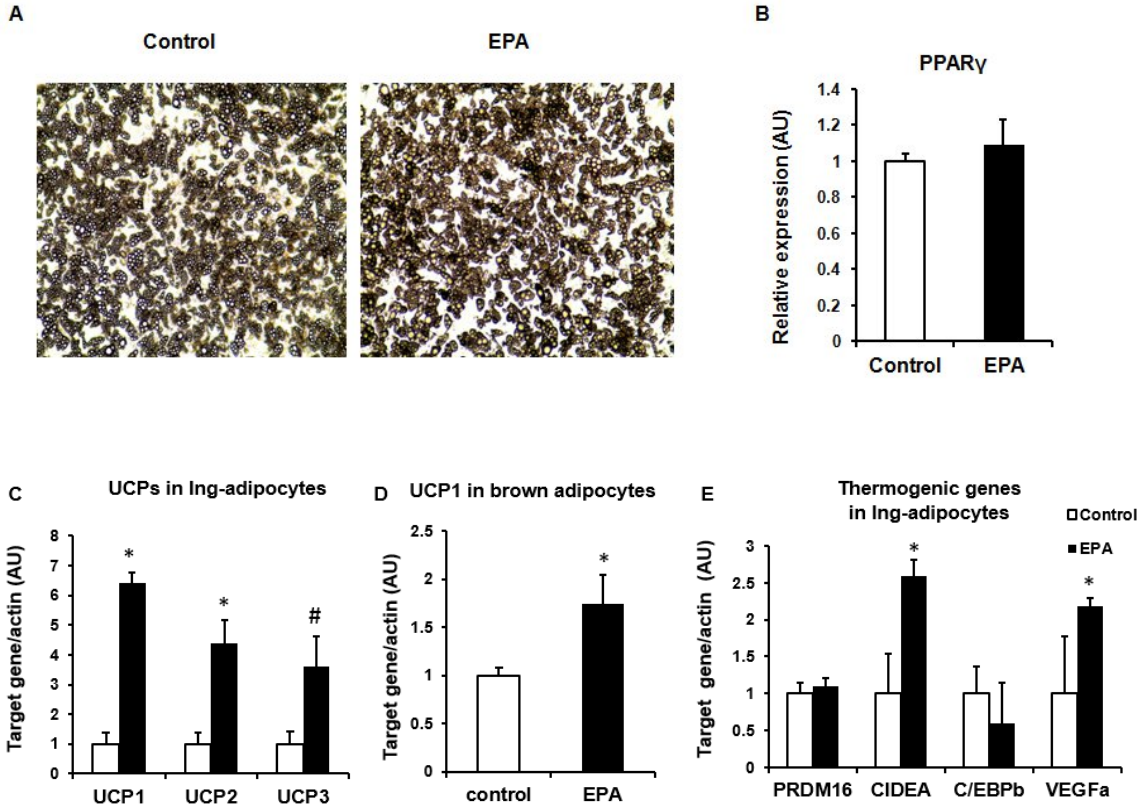


**Figure 2. EPA effect on mitochondria oxidation and glucose metabolism in inguinal and brown adipocytes.**

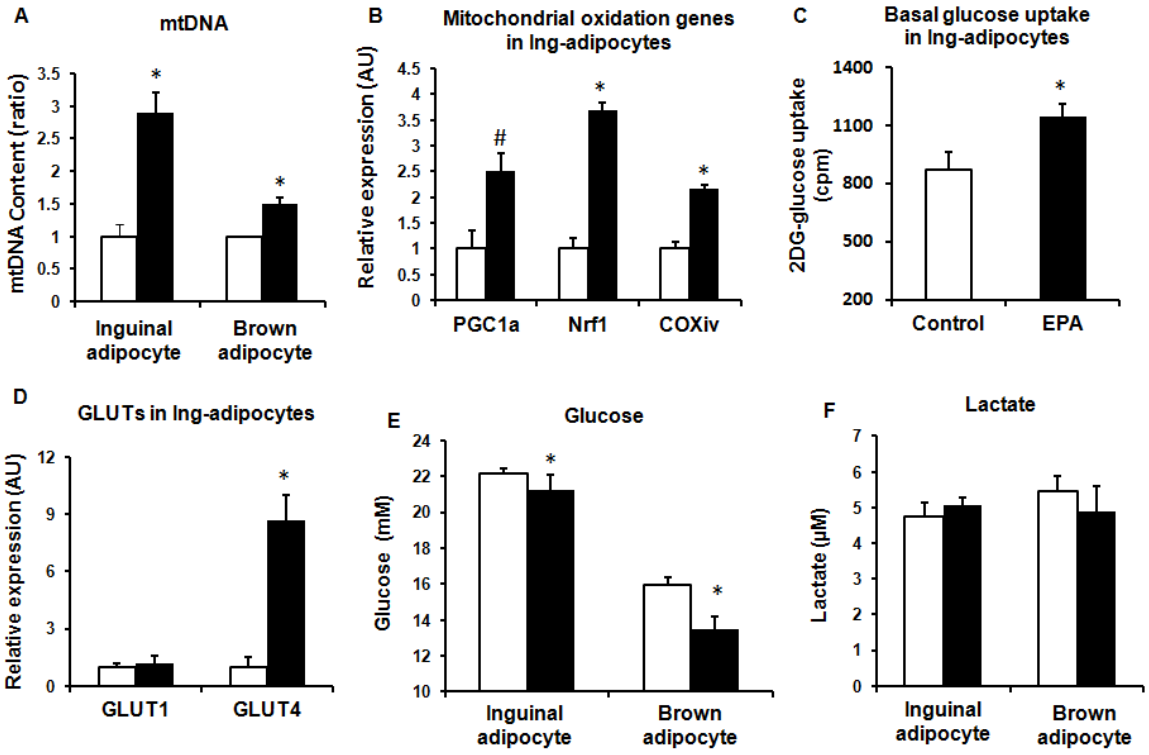
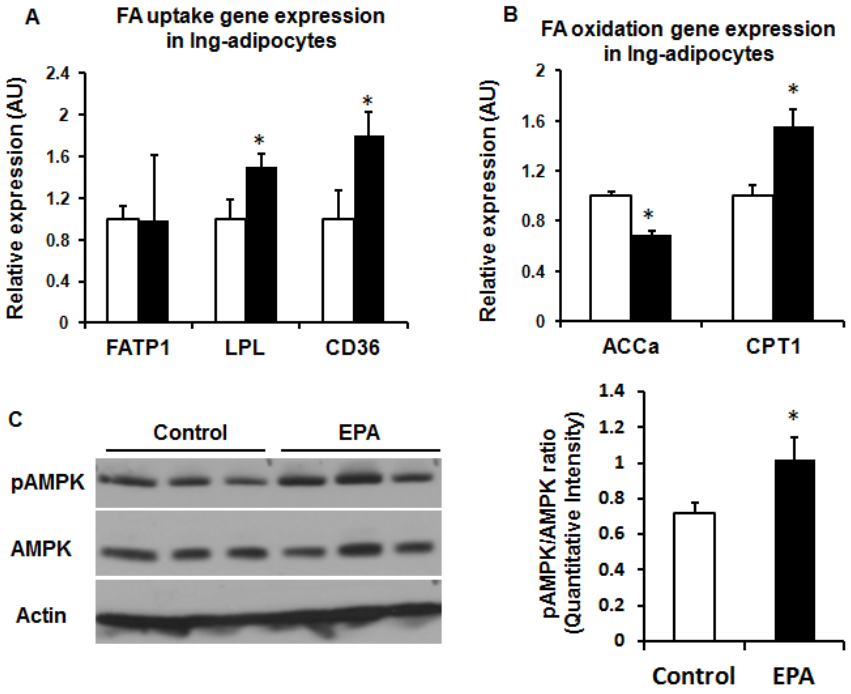


**Figure 3. EPA effect on lipid metabolism in inguinal adipocytes.**



# Figure 4. EPA effect on lipid storage in inguinal adipocytes.

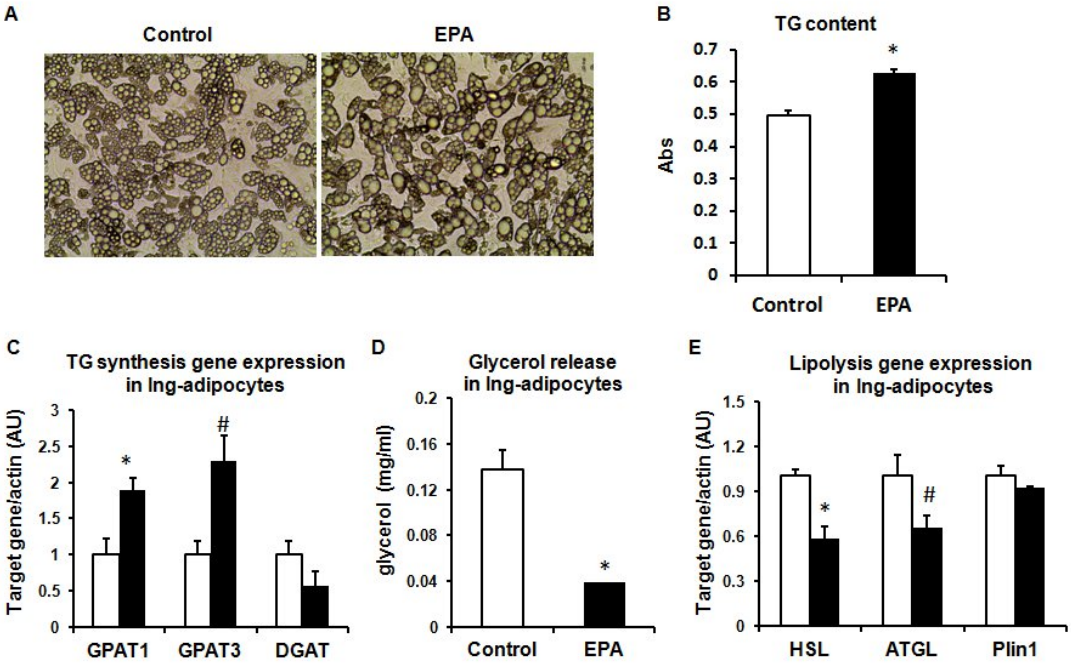
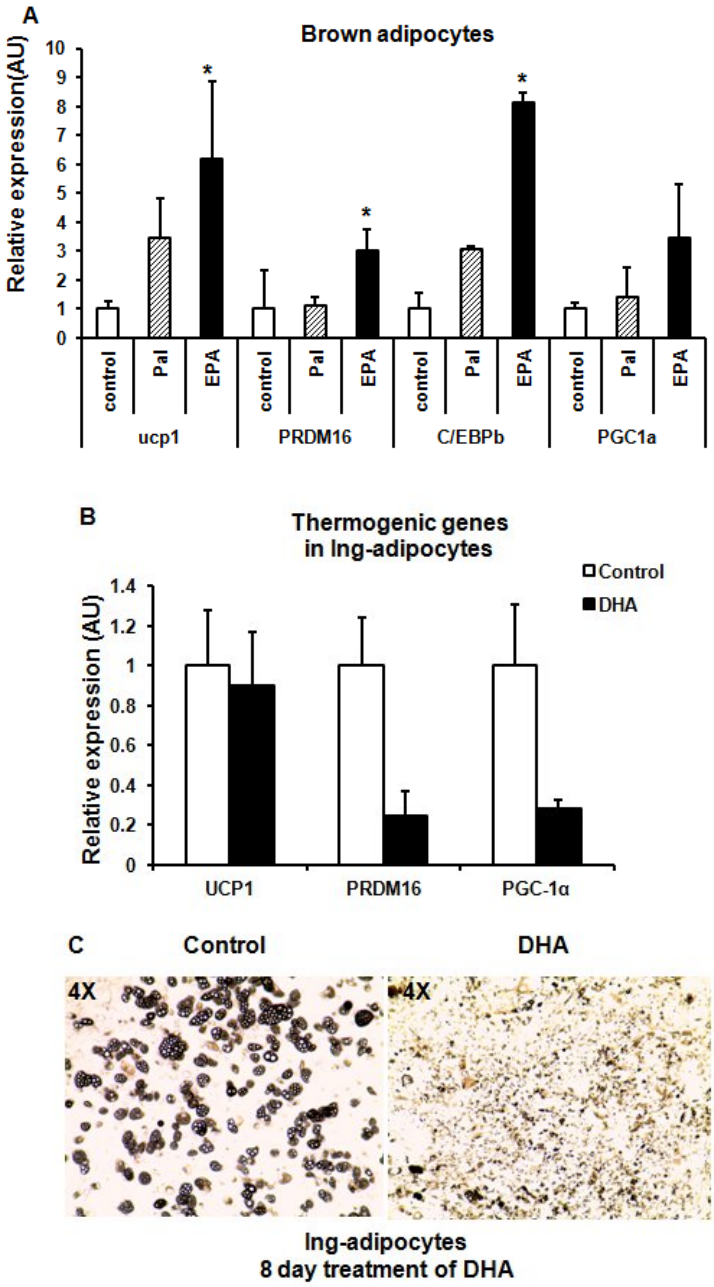


**Figure S1. Effect of palmitate and DHA on thermogenic gene expression in brown and inguinal adipocytes.**



## FIGURE LEGEND

**Figure 1. EPA effect on thermogenic gene expression in inguinal and brown adipocytes.**

Morphology of inguinal adipocytes (4X magnification) (A). Gene expression of PPARγ (B) and UCPs in inguinal adipocytes (C) and brown adipocytes (D) with EPA treatment during adipocyte differentiation. Expression of thermogenic genes in inguinal adipocytes with EPA treatment (E). The results represent 2–3 independent experiments with different sets of mice (4-5 mice). White bar: control, black bar: EPA. The data are represented as mean ± SEM. (n=3 per group) * $p<0.05$, # $p<0.1$, EPA vs. control.

**Figure 2. EPA effect on mitochondria oxidation and glucose metabolism in inguinal and brown adipocytes.**

Mitochondrial DNA content in inguinal and brown adipocytes EPA treatment (A). Expression of mitochondrial oxidation genes (B), uptake of [$^3$H]-2 deoxy-D-glucose (C), and expression of GLUT1 and GLUT4 genes (D) in inguinal adipocytes with EPA treatment. The levels of glucose (E) and lactate (F) in the culture medium of inguinal and brown adipocytes with EPA treatment. The results represent 2–3 independent experiments with different sets of mice (4-5 mice). White bar: control, black bar: EPA. The data are represented as mean ± SEM. * $p<0.05$, # $p<0.1$, EPA vs. control.

**Figure 3. EPA effect on lipid metabolism in inguinal adipocytes.**

Expression of genes involved in fatty acids uptake (A) and fatty acids oxidation (B) in inguinal adipocytes with EPA treatment. AMPK phosphorylation in inguinal adipocytes (C). The results represent 2–3 independent experiments with different sets of mice (4-5 mice). White bar: control, black bar: EPA. The data are represented as mean ± SEM. (n=3 per group) * $p<0.05$, EPA vs. control.

**Figure 4. EPA effect on lipid storage in inguinal adipocytes.**

Morphology (10 X magnification) (A) and triglyceride (TG) content (B) of inguinal adipocytes with EPA treatment. Expression of genes involved in triglyceride synthesis (C) in inguinal adipocytes with EPA treatment. The levels of glycerol in the culture medium of inguinal adipocytes with EPA treatment (D). Expression of genes involved in lipolysis (E) in inguinal adipocytes with EPA treatment. The results represent 2–3 independent experiments with different sets of mice (4-5 mice). White bar: control, black bar: EPA. The data are represented as mean ± SEM. * $p<0.05$, # $p<0.1$, EPA vs. control.

**Figure S1. Effect of palmitate and DHA on thermogenic gene expression in brown and inguinal adipocytes.**

A: SV cells were isolated from brown adipose tissue of 4-5 mice and induced to differentiate into brown adipocytes. At day 8 of differentiation, brown adipocytes were treated with palmitate (200μM) or EPA (200μM) for 24h. Cells were then harvested for thermogenic gene expression. B and C: SV cells were isolated from inguinal adipose tissue of 4-5 mice and induced to differentiate into inguinal adipocytes. DHA (200μM) was added during 8 days of adipocyte differentiation process. At day 8 of differentiation, adipocytes were harvested for thermogenic gene expression (B) or examination of overall morphologic differentiation (4X magnification) (C). The results represent 2 independent experiments. The data are represented as mean ± SEM. (n=3 per group) * $p<0.05$, EPA vs. control.

**TABLE**

## Table 1. Sequences of Primer for Real-Time RT-PCR

| Gene | Forward(5'--3') | Reverse ( 5'--3') |
|---|---|---|
| Adiponectin | GCAGAGATGGCACTCCTGGA | CCCTTCAGCTCCTGTCATTCC |
| C/EBPα | AGCTGAGTTGTGAGTTAGCCATGT | ACCCCACAAAGCCCAGAAA |
| C/EBPδ | TTCCAACCCCTTCCCTGAT | CTGGAGGGTTTGTGTTTTCTGT |
| GLUT4 | AACGGATAGGGAGCAGAAACCCAA | GTGCAAAGGGTGAGTGAGGCATTT |
| HIF-1α | TCAAGTCAGCAACGTGGAAG | TATCGAGGCTGTGTCGACTG |
| IL-1β | AAATACCTGTGGCCTTGGGC | CTTGGGATCCACACTCTCCAG |
| IL-6 | AGAAGGAGTGGCTAAGGACCAA | AACGCACTAGGTTTGCCGAG |
| LPL | TGAGAAAGGGCTCTGCCTGA | GGGCATCTGAGAGCGAGTCTT |
| MCP1 | CTTCTGGGCCTGCTGTTCA | GAGTAGCAGCAGGTGAGTGGG |
| PAI-1 | TTCCAAGGCATCCAGAAGCAGAGA | ACAGCAGCCGGAAATGACACATTG |
| PPARγ | TTGACCCAGAGCATGGTGC | GAAGTTGGTGGGCCAGAATG |
| Pref1 | CAGGCAACTTCTGTGAGATCGTA | TCGTTCTCGCATGGGTTAGG |
| TBP | GAAGAACAATCCAGACTAGCAGCA | CCTTATAGGGAACTTCACATCACAG |
| TNFα | ACG CTC TTC TGT CTA CTG AAC TTC G | ATA GCA AAT CGG CTG ACG GTG TGG |
| VEGFα | CACGACAGAAGGAGAGCAGAAGT | TTCGCTGGTAGACATCCATGAA |

| | | |
|---|---|---|
| Zfp423 | GTCACCAGTGCCCAGGAAGAAGAC | AACATCTGGTTGCACAGTTTACACTCAT |

## Table 2. Sequences of Primer for Real-Time RT-PCR

| Gene | Forward(5'--3') | Reverse ( 5'--3') |
|---|---|---|
| AdipoQ | GCAGAGATGGCACTCCTGGA | CCCTTCAGCTCCTGTCATTCC |
| C/EBPα | AGCTGAGTTGTGAGTTAGCCATGT | ACCCCACAAAGCCCAGAAA |
| Col1 | CAACCTGGACGCCATCAAG | CAGACGGCTGAGTAGGGAACA |
| Col3 | AGCTTTGTGCAAAGTGGAACCTGG | CAAGGTGGCTGCATCCCAATTCAT |
| Col4 | GGCGGTACACAGTCAGACCAT | GGAATAGCCGATCCACAGTGA |
| CtB | TGAAGAAGCTGTGTGGCACT | ATTGTTCCCGTGCATCAAA |
| CtL | CTGCTTGGGAACAGCCTTAG | GTTGCTGTATTCCCCGTTGT |
| FABP4 | GATGAAATCACCGCAGACGACA | ATTGTGGTCGACTTTCCATCCC |
| Fibronectin | ATCACAGTAGTTGCGGCAGGAGAA | TGTCATAGTCAATGCCAGGCTCCA |
| GLUT4 | AACGGATAGGGAGCAGAAACCCAA | GTGCAAAGGGTGAGTGAGGCATTT |
| IL-18 | TCTTCTGCAACCTCCAGCATC | GACATGGCAGCCATTGTTCC |
| IL-6 | AGAAGGAGTGGCTAAGGACCAA | AACGCACTAGGTTTGCCGAG |
| iNOS | CACAAGGCCACATCGGATTT | TCAATGGCATGAGGCAGGAG |
| Leptin | CCACACACAGCTGGAAACTCC | GGCTTGCTTCAGATCCATCC |
| LPL | TGAGAAAGGGCTCTGCCTGA | GGGCATCTGAGAGCGAGTCTT |
| MCP1 | CTTCTGGGCCTGCTGTTCA | GAGTAGCAGCAGGTGAGTGGG |

| | | |
|---|---|---|
| MMP9 | TATTTTTGTGTGGCGTCTGAGAA | GAGGTGGTTTAGCCGGTGAA |
| NPC2 | ATACTTGGTTGCAGGTGCTTGG | ACGCAGGAGTGGCAAGTTAGAA |
| PPARγ | TTGACCCAGAGCATGGTGC | GAAGTTGGTGGGCCAGAATG |
| TBP | GAAGAACAATCCAGACTAGCAGCA | CCTTATAGGGAACTTCACATCACAG |
| TLR2 | TTTGCTGGGCTGACTTCTCT | AAATCTCCAGCAGGAAAGCA |
| TLR4 | GGCAGCAGGTGGAATTGTAT | AGGATTCGAGGCTTTTCCAT |
| TNFα | ACGCTCTTCTGTCTACTGAACTTCG | ATAGCAAATCGGCTGACGGTGTGG |
| αSMA | ATTGTGCTGGACTCTGGAGATGGT | TGATGTCACGGACAATCTCACGCT |

## Table 3. Sequences of Primer for Real-Time RT-PCR

| Accession No. | Gene | Forward(5'--3') | Reverse ( 5'--3') |
|---|---|---|---|
| NM_133360.2 | ACCα | ATGACAGTCCTCTTCCCATGCACA | ACACTGTTCACACGACCCTATGCT |
| NM_025802.3 | ATGL | TGTGGCCTCATTCCTCCTAC | TCGTGGATGTTGCTGGAGCT |
| NM_007393.3 | β-actin | GCTCTGGCTCCTAGCACCAT | GCCACCGATCCACACAGAGT |
| NM_001159558.1 | CD36 | TCTTGGCTACAGCAAGGCCAGATA | AGCTATGCAGCATGGAACATGACG |
| NM_009883.3 | C/EBPβ | AAGCTGAGCGACGAGTACAAGATG | CTTGAACAAGTTCCGCAGGGTGCT |
| NM_007702.2 | CIDEA | TGC TCT TCT GTA TCG CCC AGT | GCC GTG TTA AGG AAT CTG CTG |
| NC_005089.1 | COX2 | TCTCCCCTCTCTCTACGCATTCTA | ACGGATTGGAAGTTCTCTATTGGC |
| NM_009941.2 | COXiv | ATGTCACGATGCTGTCTGCC | GTGCCCCTGTTCATCTCGGC |
| NM_009948.2 | CPT1β | TCTAGGCAATGCCGTTCAC | GAGCACATGGGCACCATAC |
| NM_010046.2 | DGAT1 | CTC TGC CAC AGC ATT GAG AC | TGC TAC GAC GAG TTC TTG AG |
| NM_011977.3 | FATP1 | CGC TTT CTG CGT ATC GTC TGC AAG | AAG ATG CAC GGG ATC GTG TCT |
| NM_011400.3 | GLUT1 | TCAACGAGCATCTTCGAGAAGGCA | TCGTCCAGCTCGCTCTACAACAAA |
| NM_009204.2 | GLUT4 | AACGGATAGGGAGCAGAAACCCAA | GTGCAAAGGGTGAGTGAGGCATTT |
| NM_008149.3 | GPAT1 | AGCAAGTCCTGCGCTATCAT | CTCGTGTGGGTGATTGTGAC |
| NM_172715.3 | GPAT3 | CTTTGAAATCGGAGGAACCA | TTTGCAAACTGAACTGCGTC |
| NM_001039507.2 | HSL | AGGTGGGAATCTCTGCATCACTGT | TGTCCCTGAATAGGCACTGACACA |
| NM_008509.2 | LPL | TGAGAAAGGGCTCTGCCTGA | GGGCATCTGAGAGCGAGTCTT |
| NM_010938.4 | Nrf1 | CCACGTTGGATGAGTACACG | CTGAGCCTGGGTCATTTTGT |
| NM_001113471.1 | Perilipin1 | GGCCTGGACGACAAAACC | CAGGATGGGCTCCATGAC |
| NM_008904.2 | PGC1α | ACCGTAAATCTGCGGGATGATGGA | AGTCAGTTTCGTTCGACCTGCGTA |
| NM_011146.3 | PPARγ | TTGACCCAGAGCATGGTGC | GAAGTTGGTGGGCCAGAATG |

| | | | |
|---|---|---|---|
| NM_001177995.1 | Prdm16 | AAGCTGTGCATGGCTCGTGTTTAG | AGTCACCAGGAACTGTGGTCCATT |
| AC_000038.1 | RIP140 | TCCCCGAACACGAAAAGAAAG | ACATCCATTCAAAAGCCCAGT |
| NM_009463.3 | UCP1 | ACTGCCACACCTCCAGTCATT | CTTTGCCTCACTCAGGATTGG |
| NM_011671.4 | UCP2 | TGACAAACAGGTCAAGAGAGGGCA | TCAGGCCAACTGACAGCATTCCTA |
| NM_009464.3 | UCP3 | GCAAAGCACAGGACCAACTTCCAA | ATTCTGTCCTTCCACCATGTGGGT |
| NM_001025257.3 | VEGFα | CACGACAGAAGGAGAGCAGAAGT | TTCGCTGGTAGACATCCATGAA |

# SUMMARY

In the thesis, we used adipocyte culture model to investigate regulations of inflammation and adipocyte metabolism from several different aspects. We found that LPS treatment to SV cells inhibited adipogenesis, which may due to the impairment of the adipocyte lineage commitment of adipocyte progenitor cells. We also demonstrated that endosome/lysosome protein NPC2 plays an important role in mediating LPS-induced inflammatory response. Since the structure of NPC2 is similar to TLR co-activator MD-2, we speculate that NPC2 may facilitate the binding of LPS to TLR4 in endosome, which in turn activates TRAM/TRIF pathway. In addition, we explored that EPA treatment induced the appearance of beige adipocytes features in subcutaneous adipocytes. This could increase energy expenditure, prevent inflammation and ultimately improve adipocyte metabolism. Overall, our studies expanded our understanding of the regulations of adipocyte inflammation and the consequent effect on adipocyte metabolism. Our results shed some light on the new therapeutic targets for treating adipose tissue inflammation and metabolic disorders. However, due to the limitation of time, we couldn't study all the details of the mechanisms. Further studies are needed to verify our hypothesis that arose from the observations in these studies, such as the mechanism by which LPS disrupts adipocyte commitment and the role of NPC2 as a co-activator of TLR4 in endosomes.

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